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THE MUSCULATURE OF THE BRONCHI AND LUNGS

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The fact that the lungs are muscular organs, in a very real sense, has become increasingly obvious in recent years. To such an extent, indeed, has this conception made headway that one modern worker, Baltisberger (18), after carefully studying the human lung, has felt safe in asserting that hardly a cubic millimeter of pulmonary tissue exists which does not contain smooth muscle, and others have made statements almost as striking. Organized, as it is, into a very complex system, this muscle would seem of the utmost functional importance—in fact quite indispensable in respiration. Considered as a whole, it falls naturally into two great anatomical divisions (18): the first, the more evident and well recognized, is related to the airway, and is known usually as the bronchial musculature; while the second occurs in scattered wisps through the interstitial tissue and in the deeper layers of the pleura, and is referred to as the interstitial musculature.

The bronchial musculature, proper, has been a subject of investigation and speculation for many years. Physiologically it is much the more important division, and its discussion will occupy most of the present paper. It may be objectivized as a contractile net, embracing the mucosa of the entire bronchial tree, from the larynx to the alveolar mouths, whose strands, though stout enough to be easily seen with the naked eye in the trunk and larger branches, become more and more delicate as we trace them down through the finer tubes into the terminals, where they are of microscopic size. It is, when considered as a whole, a replica of the bronchial tree, whose form we would have preserved accurately in muscle were it possible to dissolve away all of the other tissue. To visualize the bronchial musculature, then, we must first have a clear-cut concept of the entire bronchial tree, including not only its trunk and large and small branches, but particularly its twigs

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and leaves, if we may use this term (488) to designate the ultimate respiratory chambers.

THE ANATOMY OF THE BRONCHIAL TREE. The bronchial tree, for purposes of description, will be divided into two parts. The first is the purely *conducting*, central region, comprising the trunk and its branches, which may be likened to a tree minus the leaves; thus it embraces the entire air-tube system from the trachea to the terminal bronchioles, inclusive. The form of this division has become familiar from dissections, and particularly from casts of the lumen, such as those shown in the atlas of Spalteholz (405).

The second division is the combined *conducting and respiratory* portion, its branched tubes being characterized by mural outpouchings lined with respiratory epithelium. It includes all of the airway lying distal to the terminal bronchioles. Like the leaves, it represents the peripheral region of the tree, for its units begin as direct continuations of the end-twigs of the first part. This will be referred to as the *respiratory* division. While acknowledging the distinctiveness of these two parts, it will be well to remember that the bronchial tree, with all its ultimate ramifications, is an anatomical and physiological unit, including, indeed, with its vascular, nervous and supporting adnexa, the entire lung substance. However, the above method of division seems to find an additional support in the peculiarity of the blood supply, for the conducting airway, from the main bronchi to the terminal bronchioles, inclusive, is served by the bronchial arteries (239, 253, 297, 298, 314, 318, 396) while beyond this point, i.e., in the supply of the respiratory airway, the pulmonary artery is the source. Although there are no true anastomoses between the two systems (154, 314, 318) there is extensive union between their respective capillary beds in the respiratory portion of the airway. The pleura, with its musculature, receives its blood sometimes from the pulmonary artery, as in the dog; sometimes wholly from the bronchial artery, as in the horse and man, and sometimes principally from the bronchial artery, as in the sheep (300, 301). For a detailed description of the blood supply to the bronchial musculature, see Miller (318).

The *conducting* part of the tree is simply a system of tubes for distributing the air to and collecting it from the innumerable respiratory units, or pulmonary lobules. From the trunk, or trachea, the entering current flows through the two main bronchi, and their branches of progressively increasing number and diminishing size, into the multitudinous fine terminal bronchioles, which are the extremities of the purely conducting

part of the tree. Thence the entering air passes as a myriad of thin jets into the respiratory bronchioles, and on through the ramifications of these into the ultimate subdivisions of the pulmonary lobules. Through this complexly ramifying hollow tree there is a constant flowing and ebbing of air to and from the respiratory "leaves." The area of cross-section of the branches is always greater than that of their parent stems (194), so that the entering air current is progressively widened and slowed.

The morphology of the *respiratory part* of the airway has presented a difficult problem to anatomists for many years. The discrepancies of opinion concerning this are referable largely to the inadequacies of the methods adopted for its investigation. Single sections are practically useless; casts of the lumen, said to have been first made by Bidloo, are not only deformed, due to the distention and obstruction occasioned by the air entrapped in the terminal chambers, but, if the injection be complete, they are practically solid masses on account of the closeness of interdigitation of the parts of neighboring lung acini, and the thinness of the partitions separating their end-compartments. These structural peculiarities make it impossible to gain much information from fragments of lung tissue which have been injected with opaque fluid masses and subsequently cleared. Carefully prepared models made from serial sections are instructive, but on account of the complexity of the architecture, as well as the difficulties and tediousness of the method, it is necessary rigidly to limit the attempted reproduction to very simple details. These, as well as other reasons, account for the regrettable lack of uniformity in the anatomical descriptions of the structure of the smaller divisions of the bronchial tree. However, the immense advance made so far gives us hope that, with our improving technique, it may not be long before we are in possession of a true picture of this very interesting fragment of Nature's handiwork.

To set forth in detail the evolution of the conceptions of the finer terminals of the airway is beyond the scope of this paper, and reference must be made to articles more especially devoted to this subject: (76, 188, 208, 225, 227, 240, 262, 292, 293, 294, 295, 296, 304, 307, 308, 309, 312, 313, 315, 317, 319, 320, 327, 332, 333, 335, 367, 376, 394, 395, 463, 471). It is apparent, from a survey of the literature, that anatomists have progressed in the last century from ideas of very simple structure to those involving great complexity.

The many terms introduced by the numerous authors to indicate identical subdivisions of the airway have served further to obscure the

subject. Miller (295) gives a table of the designations used up to the year 1900 for these various branchings. "Bronchus III," "bronchiolus respiratorius," "bronchial tube" and "alveolargang" referred to the same structure; "terminal bronchus," "ductulus alveolaris" and "lobular bronchus" were applied to the next subdivision: "atrium," a term introduced by Miller (292) to designate that portion of the tube which he described as an expansion intervening between his "terminal bronchus" and the air sacs, several of which opened off from one atrium, was next in order. Finally, the wall of the "air-sac," "infundibulum" or "alveolar-sac" was subdivided into the "air-cells," "alveoli" or "alveoli pulmonis." It is not claimed that this list is complete. The same term has been used with different meanings by different workers.

At present "terminal bronchiole" is the name most current among authors to designate the last portion of the conducting airway (188, 262), and will be so applied here. It is the terminal and finest subdivision of a system of repeatedly branching bronchioles. "Respiratory bronchiole" will refer only to the first tubes bearing alveoli in their walls. They arise immediately from the terminal bronchioles. Springing from the respiratory bronchiole is the "alveolar duct," which opens into the "alveolar sac," in the walls of which the "alveolus" is the final unit. These are the only terms which will be used in this review.

"Atria" were first described by Miller (292, 295, 299, 304), and his views were supported by Justesen (208), but other workers either failed to find them (327) or considered them as parts of the alveolar ducts (395), while others (332, 333, 471) deemed the term unnecessary or denied the existence of such a subdivision for the human lung. I have in my studies been unable to convince myself that the atrium is a clear-cut constant entity, and there are undoubtedly many alveolar sacs which leave the alveolar duct without the interposition of a dilated antechamber. I shall not use the term until the existence of the structure to which it refers is more generally accepted.

The term "infundibulum," first said to have been used (471) by Rossignol (376) to describe the most distal division of the bronchial tree, is a misnomer, for it suggests a structure having the shape of a funnel, which is obviously not that of the alveolar sac in its entirety. Many workers (as 18, 335) have advocated its discontinuance.

There seems to be some confusion as to just what is meant by an "alveolus," some writers including under this term spaces of varying size, divided up more or less completely by septa into two or more smaller cavities, while others would apply it only to the ultimate res-

piratory chamber, the finest subdivision of the alveolar sac, as I have done.

Another point of dissension is the question of how much is to be included in the "lung unit," termed "acinus" by some (188, 262), and "primary lobule" by others (307, 308). Loeschke conceives the acinus to be all that tissue supplied by one terminal bronchiole, while Husten would include only half this amount, or all that lung substance including and beyond the respiratory bronchiole, of which there are two branching from one terminal bronchiole. Miller defines a primary lobule as an alveolar duct and its offshoots, with its blood and nerve supply and supporting tissues, though it would seem that the lung unit, even when his definition is accepted, is far more complex than his plate I (308) would indicate. He feels (307) that if "acinus" is used in describing any part of the lung it should be applied to the atrium and its alveolar sacs.

This mooted point cannot be settled here, but it would seem that Husten's definition of the lung unit, the "acinus" or "primary lobule," is most nearly in harmony with our conception of that term as applied to glands, where it is commonly understood to include only that secreting tissue which lies beyond the ducts. The respiratory bronchiole is the first part of the tree in which respiratory epithelium is present, and although the amount of gaseous exchange may be slight in this short length of tube, it represents the beginning of the tissue in which essential lung function is carried out. Hence it, and all that tissue branching off from it, may be regarded as composing a single anatomical unit.

It is of interest that Nicol (330) finds, in the very early stages of tuberculosis, that the foci correspond in distribution to this anatomical unit, that is, a respiratory bronchiole and all its ramifications. Gross (152), too, finds that in animals subjected to dust inhalations over long periods of time, the particulate matter settles in scattered foci, which correspond in form and distribution to the lung acinus. The factors which are at the basis of this unequal distribution are not well understood.

The caliber of the respiratory bronchiole is the same as that of the terminal bronchiole from which it arises, or may be greater than this (188, 262); indeed it is usually widened toward its distal end. Small outpouchings from the wall, covered by thick cuboidal epithelium at times, and at others by respiratory epithelium, project between the meshes of the bronchial muscle, at first only on that side of the bronchiole opposite the accompanying pulmonary artery (18). As we pass periph-

erally, the sacculations become more and more numerous, and occupy collectively an increasing proportion of the mural area, thus reducing the thickness of the intervening meshwork, while at the same time they gain more and more of the characters of true alveoli. Even alveolar sacs may arise from the sides of the respiratory bronchioles (188). The tubes branch, passing insensibly into alveolar ducts of about the same, or somewhat larger, caliber.

The alveolar duct is often pictured as a relatively simple tube having walls from which emerge only simple alveoli, the alveolar sacs being shown only at its termination. This conception is quite inadequate—indeed erroneous—and it seems possible that one of the sources of the false idea that alveolar sacs are to be found only at the extremities of the alveolar ducts is a common method of preparation of lungs used for such studies. This is the inflation and subsequent drying of lungs removed from the thoracic cavity. Sections of these often show the subpleural alveolar sacs much distended and standing out very plainly, so that attention has been concentrated on them to the neglect of the less conspicuous but infinitely larger number of alveolar sacs situated in the interior of the lung. So a false impression has been conveyed which text-book figures (as fig. 61d, 288) have served to perpetuate.

The alveolar ducts, then, bear in their walls not only alveoli, but also alveolar sacs of varying size, and they branch and rebranch into other alveolar ducts. It is likely that the alveolar sacs are, as compared with the alveoli, relatively more numerous toward the peripheral ends of the ducts than they are in their proximal parts, and they are probably also larger and more complexly subdivided in the outlying part of the duct. Thus the alveolar duct may be visualized as a branched tubular net, through the meshes of which air is flowing alternately into and out of the outlying respiratory chambers.

According to Miller, the lymphatics of the bronchial tree do not extend past the alveolar duct, the alveolar sacs and alveoli possessing no lymphatics within their walls (294, 305, 306, 311). The flow from these is toward the hilus of the lung, although those which exist in the septa drain into the pleura (76) or into the pre-aortic lymph nodes (84).

As Kölliker (225) and others have observed, the finer terminals of the mammalian lung are very similar to the entire lung of the frog. When the latter is fixed in the inflated condition and slit open, and the interior examined with a binocular microscope, one beholds, as it were, a mammoth alveolar sac, or perhaps more accurately, a short, much dilated, alveolar duct. The entire inner wall is composed of a contractile net-

work, through the meshes of which the air passes to and from the respiratory chambers, which, in turn, are further subdivided into smaller compartments by lower crescentic ridges whose edges often run up to and merge with the contractile mouth of the main chamber. If now one visualizes many such lungs, built upon a much reduced scale, fitted compactly against one another, their outpouchings interdigitating, and all opening out into a common, long, branched passage, one has a fairly clear idea of the relations between alveolar sacs and alveolar ducts.

An excellent method for the study of the respiratory portion of the airway is the following, adopted by the author and A. Gordon Calder. A healthy adult cat was chloroformed and bled through the inferior vena cava, into which a 10 per cent formalin solution was then injected under gentle pressure, thus fixing the lungs *in situ* in the phase of expiration, before the thorax was opened. The lungs were allowed to stand thus for twenty-four hours, when they had become stiff enough to retain their form. They were then carefully removed, without crushing, and allowed to remain immersed in the same fixative. After washing in water, pieces were cut out with a thin razor blade and hardened in ascending alcohols, stained with carmine and attached to the bottom of Syracuse watch glasses with celloidin while immersed in 80 per cent alcohol. Dissection with sharp iridectomy knives, under a binocular microscope, was then carried out. It was possible to begin with a terminal bronchiole, and follow its branches to their outer extremities.

The picture presented was striking indeed. The terminal bronchioles were obviously narrow, and their inner walls bore longitudinal ridges, which became changed, farther out, into a netlike structure. Depressions, small at the proximal end of the tube, and larger and more numerous distally, began to appear between the ridges until, when the true alveolar duct was reached, the wall was nothing but a network composed of the thin boundaries of the rounded openings leading into the chambers already described. This delicate meshwork collectively forms the contractile net already mentioned. The lesser openings led into small alveolar sacs, which were further partially subdivided by low partitions into two or more alveoli, the number being in proportion to the capacity of the sac in whose walls they were situated. There was much variation in the height of the partitions separating these alveoli, and in the size of the air cells. The wider openings transmitted air to and from the more voluminous alveolar sacs, and the largest of them led into branches of the alveolar ducts. The principal element of this contractile net is a myoelastic tissue—a combination of the fine strands of bronchial smooth muscle intermingled with delicate fibers of yellow elastic tissue.

If one would gain some conception of the complexity of the human lung unit without actually studying the specimen itself, a glance at figure 3 of Willson (471) will be of help. His description of this part of the airway gives, to my mind, the best impression of its very intricate character. In this figure a terminal bronchiole is shown dividing into two respiratory bronchioles, each of which divides into two alveolar ducts. Only one of these was followed by Willson, and this divided twice in rapid succession. One only of the latter branches was traced to its ultimate terminals. These ramifications were all projected upon a sheet of paper. On this branch and its ramifications alone one can count some seventy-one alveolar sacs of various sizes, to say nothing of the many small alveoli. Thus the number of respiratory chambers in a single pulmonary lobule must be enormous, for only a very small proportion of them could be illustrated in this way. Willson noted much variation in the size of the alveoli, and of the air sacs, and found many respiratory chambers which might with equal propriety be called either large alveoli or small air sacs.

Anastomoses between branches of the bronchial tree do not occur, as Reisseisen (364) pointed out, although Miller (320) has found that they may appear rarely in the human lungs as "fetal anomalies."

This lung lobule, built up as above described, is evidently the essential expansile part of the lung (217). It is a complicated, many-branched "bellows part," as Keith (217) terms it; indeed this is the structure which he speaks of physiologically as the "infundibulum." It will now be our task to examine the bronchial musculature at length, fitting it into the complicated system which we have just outlined.

ANATOMY OF THE BRONCHIAL MUSCULATURE. Although, as stated, the bronchial musculature is a continuous system, reproducing the form of the bronchial tree from the root to the leaves, it will be convenient to discuss it in relation to its aforementioned conducting and respiratory divisions.

At the outset it may be said that, until the contribution of Reisseisen (364) in 1822, the conceptions of this system were very vague indeed. This patient and thorough investigator, after whom the muscle is named, carried on researches which laid the foundation of our knowledge of its structure. His method was the dissection of fresh material with the aid of simple hand lenses, and it is astonishing that with such primitive means he was able to trace this muscle coat down to the finer bronchioles. He inferred that this muscle-system continued to the ends of the air-tract, and this has since been confirmed. His figures are still not only

objects of interest, but of much value, and although the picture he presented of the system lacks completeness with respect to its terminals, yet it stands, perhaps, as the greatest single contribution to the subject under discussion.

The problem has since been attacked by a large number of workers, and latterly the interest has centered around the muscle of the respiratory part of the airway, where outstanding contributions have recently been made by Miller (313) and Baltisberger (18), among others.

Fundamental plan. The fundamental plan of the bronchial musculature is the same throughout the bronchial tree; indeed it is the same, in a larger sense, in all animals which possess it, though there are certain minor peculiarities in the different species. As pointed out by Reisseisen, its structure is such as to permit easily of adaptation to the rhythmic length and width changes which he recognized to be an integral part of the processes of lung inflation and deflation; indeed to take an active part in these movements. This structure may be described as a branched, tubular net. The fasciculi are arranged neither as a series of transverse circles, nor even less as parallel, longitudinally coursing fasciculi, but the fabric has to a certain extent the properties of both plans, combined with advantages of its own. It should be always remembered that the muscle is being discussed here, as far as possible, with the lung in the inflated condition—as it is, for instance, when fixed before removal from the thorax. If the lungs are allowed to collapse, the bronchi all shorten, and this has the effect of approximating the strands of muscle and of closing up the meshes of the net. Here we have, probably, the reason why some authors describe the coat as being completely closed (391), (444-bronchi of cat), and made up of circularly-coursing fasciculi (129, 394). It may be said, too, that the meshes of the net are relatively narrower in the larger tubes than they are in the smaller—that as we proceed toward the extremities of the tree there is a tendency for the strands to course more and more obliquely; indeed there is a rather sudden transition, in this respect, in the respiratory bronchioles, where the alveoli in the walls alter the muscle pattern. All through the respiratory part of the airway the muscle fasciculi course as much longitudinally as they do transversely.

There is, as might be expected, in general a direct relation between the size of the tube and the strength and stoutness of the muscle fasciculi, but this is by no means absolute, and we find local variations in the relative thickness and development of the muscle as compared with the other structures composing the wall. Thus there seems to be a tendency

for the muscle coat, although actually becoming more and more delicate as it is traced from the wider into the narrower tubes of the conducting division, to increase in stoutness as compared with the other constituents of the wall, until in the "terminal bronchiole" it is a relatively conspicuous structure.

The efficiency of action of the muscle is necessarily correlated with its histological relations with the other constituents of the bronchial wall, and we find that these relations are very constant and definite. The coat lies between the cartilage layer and the mucosa. The epithelial layer of the mucosa has to be sufficiently extensive to cover the wall in its most stretched condition, so that, when the tube is contracted it is thrown into folds or rugae. It is very common to find these folds in histological specimens, because these are usually from bronchi whose walls have contracted, not only on account of fixation after collapse of the lung, but also because of the shrinking action of the fixative on the muscle.

The tunica propria is loose, vascular, and infiltrated with wandering cells and lymphocytes (302, 308), which increase with old age (316). It is traversed by nerve fibers. Its most prominent constituent is the "elastic membrane" (280), situated upon the inner surface of the bronchial muscle coat. This layer of longitudinally coursing, branched elastic fibers arises in the region of the larynx and passes uninterruptedly from this point to the farthest alveoli. In the respiratory part of the airway the strands pursue a wavy, rather than a straight course, following the muscle. The tunica propria is admirably constructed to adapt itself to the respiratory width and length changes of the airway.

The bronchial muscle coat is itself intermingled with a loosely woven layer of elastic fibers arranged mainly circularly, in line with the muscle fasciculi (289), of which, physiologically, they may rightfully be regarded as an integral part. They are quite conspicuous, in Weigert preparations, though in comparison with the elastic membrane their formation is delicate indeed. There is here a splendid example of a tissue partnership between smooth muscle and elastic tissue in the interest of a common service, which we find in so many places, as in the blood vessels, particularly the arteries, and also in the intestinal tract, spleen and other organs. So rich is this admixture of elastic tissue in the bronchial musculature that it must be considered as an indispensable element, and the tissue is properly termed "myoelastic."

This layer of elastic fibers in the muscle has been described, along with other layers of elastic tissue in the bronchi, including the elastic

membrane, by many workers: (2, 24, 35, 40, 47, 48, 49, 71, 72, 86, 104, 114, 129, 133, 157, 225, 229, 231, 233, 242, 243, 244, 252, 255, 259, 280, 289, 338, 357, 362, 364, 387, 401, 402, 430, 431, 432, 444, 445, 470, 479). Strands from this elastic layer pass to the elastic membrane, internally, and to the elastic tissue of the adventitia, externally. In the respiratory part of the airway it is represented by the elastic strands interwoven with the muscle-net.

Intertwined with the muscle-system is a rich nutrient network of blood vessels. These, in the conducting part of the airway, arise from the bronchial arteries, whereas in the respiratory part they come from the pulmonary artery branches (318). A capillary anastomotic system between vessels from these two sources exists in the region of the respiratory bronchiole.

Between the muscle layer and the outlying cartilage there are frequent mucous glands, whose ducts pierce the muscle. Enwrapping these structures is a loose adventitia, made up of both collagenous and elastic fibers, and carrying blood vessels and nerves. This tissue, except in the lung roots, passes over into the delicate stroma of the lung, and forms the basis of a multitude of alveoli. It is obvious that these adapt themselves easily, by stretching, to the length and caliber changes of the bronchi.

In the smaller, non-cartilaginous bronchioles the muscle is outwardly bounded by an adventitia, rich in elastic tissue, which passes over into the alveoli. Here the tunica becomes very thin, and farther on, in the passages of the respiratory division, the elastic membrane becomes broken up by the alveoli of the wall, and blends with the muscle to form sphincters for the annexed alveolar sacs and alveoli.

It will be well, now, to discuss the local structural modifications of the bronchial musculature.

Trachea. It has long been a matter of interest that the trachea should have a posterior flaccid segment. This was at one time thought to be for the purpose of facilitating the passage of a large bolus of food down the esophagus by making it possible for the adjacent tracheal wall to be pushed in, but it was realized by Monro in 1795 (303) that this was a very imperfect explanation, since the esophagus does not always lie in contact with this membrane, and since the main bronchi also show the same structure. This strip is undoubtedly contractile, and contains as its most conspicuous element the trachealis muscle, whose fasciculi run almost exclusively transversely. Occasionally there are a few longitudinal fibers, scattered mainly in the region of the carina (303). More

frequently they pursue an oblique or arciform course. The transverse strands connect the ends of the tracheal cartilages, in some animals being attached to the inner aspect of these, as in man, whereas in others, as the rabbit, they are affixed to the outer edge (303). The bundles are frequently branched, and often link together cartilages of different levels, thus leaving no gaps in the membrane, but forming a muscular net, the meshes of which can open out when the trachea is elongated, as in inspiration (281), or when the head is thrown backwards, or in peristaltoid movements (363). At the bifurcation the fibers curve downward from the sides to become attached to the carina, sometimes from cartilages situated at a considerably higher level. When this muscle-sheet is contracted the mucosa, particularly that against which it lies, is thrown into longitudinal folds, which project into the lumen. It seems reasonable to think of the open "rings" of cartilage of the trachea and main bronchi as operating like weak springs to keep the membranous posterior wall taut, and thus they would act as antagonists of the muscle-layer.

Main bronchi. In the main bronchi the structure is at first the same as it is in the trachea. As we pass to the stem bronchi, the changing form of the cartilages is accompanied by a modification of the muscle pattern. The cartilages lose their regular horseshoe shape, becoming irregular crescentic plaques, and there ceases to be an open space posteriorly. The muscle gradually extends to embrace the entire tube, relinquishing its direct attachment to the cartilage, and approaching the usual formation of circular smooth muscle. Indeed it has been described as comparable to the circular muscle of the intestine (384, 444), and has been likened to a muscular tube with only occasional breaks in continuity (129). These conceptions are not strictly accurate; the intestinal circular muscle is a compact, relatively thick layer, while that of the bronchi is open-meshed and inconspicuous in comparison. In spite of its delicate structure, it forms an important component of the bronchial wall, and its mass, when compared with that of the other mural elements, increases as we pass from the larger to the smaller branches of the tree.

The muscle strands, too, are disposed not in true circles, but rather in spirals and "geodesic" lines (313), and are interwoven in a network, the elongated meshes of which, in the larger tubes, have a transverse long axis and pointed ends. Branching of the fasciculi is frequent. Often the appearance is that of adjacent meridians on a globe. Thus, in cross sections, it is the rule to find obliquely cut pieces of the bundles, rather than entire circles, especially if the bronchus has been hardened in the inflated condition.

Stem bronchi and branches. Although the same general form of the muscle-net is maintained, there is a progressive diminution in the thickness of the strands as the tubes become reduced in caliber. As before, a transverse section rarely reveals the muscle in complete circles, although here, too, it has been described as a compact circular layer (288, 391, 394), an appearance referable to collapsed condition of the lung, or to some special type of animal used. At the point of branching of the bronchi, the muscle bundles arch from the sides of the tubes to become attached to the apex of the keel, thus providing for movement at this point in correspondence with that of the rest of the wall.

Bronchioles. As we pass into the bronchioles the muscle, adhering to the same morphological plan as in the larger tubes, becomes relatively more conspicuous, when compared with the size of the tube (189, 313), and its function may be looked upon as proportionately more important. In the human bronchiole Miller (313) reports the bands as being 0.02 mm. to 0.03 mm. in thickness, and from 0.008 mm. to 0.05 mm. in width. Among the older authors there were those who regarded this part of the airway as surrounded by a compact circular muscle layer (81, 156, 369) —an idea probably arising from the study of contracted bronchioles, though possibly also from the type of animal used, the guinea pig, for instance, having a bronchiole very richly endowed with muscle. Miller's (313) figures are very instructive, particularly those from his models of the muscle-net on the airway terminals. Where branches occur their origins are enlaced by crossed muscle strands. These models were from lungs fixed in the condition of full inspiration.

The terminal bronchiole, whose lumen is the narrowest in the entire bronchial tree (188, 262), and whose musculature is relatively the most strongly developed (189), may be completely obliterated by contraction of its walls. It is probable that this is the site of the spasm which occurs in some animals in anaphylactic shock, and in asthma, although the point has been located more centrally in the former condition, in the secondary and tertiary bronchi (393); and more peripherally in the latter disease, at the junction of the alveolar duct and "atrium" (313, 316, 318). Considering the fact that only a fraction of the respiratory surface is needed to carry on adequate oxygenation (according to Loewy and Zuntz's figures—408—only one-twentieth is utilized during rest), and that a very large proportion of the alveolar epithelium lies central to the most peripheral alveolar sacs (see figure 3, of Willson—471), it seems doubtful if a constriction located so far out as the position of the hypothetical atrium could produce so profound an asphyxia as one finds

in many cases of asthma. It would be curious, too, if these terminal muscle-wisps should be involved in spasms of constriction without the main muscle-mass being affected, and Miller (313) thinks it not improbable that muscle spasm of the respiratory bronchioles and alveolar ducts is associated with that of the "atrial" sphincters.

The musculature of the respiratory part of the airway. With the conception before us of the form of the terminations of the airway recently outlined, we may discuss the question of the musculature of this region. It is a little difficult to convey, accurately, the various opinions which have been expressed, for the ideas of the earlier workers as to the form of the terminals of the airway were very imperfect.

The muscular network is continued from the terminal into the respiratory bronchioles, gradually becoming more delicate. The muscle fasciculi in the wall of the latter tube surround the mouths of the alveolar outpouchings, and often the strands will encircle as a sphincter areas which are not actually sacculated, but which are merely filled with a membrane of epithelium (18). At such points Miller (313) states that the muscle is to be regarded, not as a part of the alveolar wall, but as an integral part of the wall of the bronchiole.

As we pass into the alveolar duct the muscle becomes both relatively and actually much less dense than in the respiratory bronchiole (18). The meshes between the muscle strands are very wide (394), the lumen of the mesh being occupied by the large openings leading into other alveolar ducts, into alveolar sacs, or into alveoli. Longitudinal sections, following the axis of the alveolar ducts, show many of the fibres of muscle cut directly across, but most are severed obliquely, and the variety of slant is almost infinite. These muscle-bundles present a curious and characteristic appearance in the sections, and are well shown in Baltisberger's figures (18), where they appear as knobs at the end of thin partitions, which seem to be projecting into the lumen, but which are in reality merely the walls of adjacent respiratory chambers opening into the duct through the sphincters, sections of which are seen in the knobs. These formations have proved puzzling to many histologists.

In cross sections of the duct the muscle is rarely seen in circles, but occurs most often as oblique sections of the fasciculi. If a complete circle is found the fasciculi are observed running in different directions in it (18); it is not a true circular layer. Compared with the respiratory bronchiole which precedes it, the alveolar duct would seem to be capable of a greater range of dilatation and contraction. Lengthening and widening of the duct leads to widening of the sphincteric meshes in its walls.

Thus increase in the dimensions of the alveolar duct, as in inspiration, serves to open still wider the entrance into the respiratory chambers.

The muscle-net of the alveolar duct, like that of the whole bronchial system, contains numerous elastic fibers whose direction is parallel with that of the muscle-strands (394). They represent, apparently, the combined attenuated layers known as the elastic membrane and the layer intermixed with the muscle, described in the larger tubes. These serve to impart a steadiness and evenness to the dilatation process, and contribute materially to the recoil mechanism. Thus the opening of each alveolar sac of the mammalian lung, like that of the lung of the frog, is encircled by a ring of myoelastic tissue, in which smooth muscle is the predominant element, intermingled with elastic fibers which collectively form a ring, or, more graphically, a wreath, and with a reinforcement of white collagenous fibers, often spoken of as reticulum. The last mentioned fibers, in the usual preparations, are of wavy outline, but it may be assumed that the lines straighten out in full inflation. Hence we may well speak of each alveolar sac as having as its inlet a myoelastic sphincter. Although smooth muscle, as in the lung of the frog, is found in the edges of the larger septa subdividing the alveolar sacs, it is rare in the smaller partitions, which are usually suspended and kept taut by beautiful arcs of elastic fibers strengthened by a few delicate white fibers of reticulum, appearing most often as exceedingly fine, wavy lines.

There has been a great deal of discussion as to just how far the muscle extends toward the periphery of the bronchial tree. Some workers have averred that it is not to be found beyond the region of the respiratory bronchiole (427); others have noted it in the alveolar ducts (18, 97, 103, 225, 227, 295, 312, 313, 394); still others have reported it as occurring in the walls of the alveolar sacs (18, 177, 369), and some workers state that it is even present in the wall of the alveolus (101, 138, 322, 323, 346, 369). Kölliker (224) originally supported this view, but later (225) reversed his decision. Miller (313) thinks that these positive findings are due to peculiarities in method of sectioning the tissue, for a section through the mouth of an alveolus might easily contain muscle which rightfully should be regarded as belonging to the airway, and not to the alveolus. The prevalent opinion now is that the wall of the alveolus, proper, contains no muscle. Baltisberger (18), in a recent paper, holds that muscle, in the human subject, occurs frequently, though not always, in the edges of the septa which subdivide the alveolar sac, and this agrees with my own observations in the lung of the cat.

This ground should all be re-worked by the comparative histologists, who should study the muscle development in the light of the varying habits and specialized physiology of the animals. Variations occur in actual density and in relative density in different parts. Why, for instance, is the muscle so well developed in the guinea pig (313)?

THE PHYSIOLOGY OF THE BRONCHIAL MUSCULATURE. Having obtained a conception of the form and relations of the bronchial musculature, and its arrangement in the different parts of the bronchial tree, we may discuss the physiology of this system, enquiring as to its active participation in the respiratory movements of the airway, and in special happenings, such as the expulsion of exudate. As we have seen, the mode of attachment and the disposition of the various parts of this muscular organ permit of much flexibility in the air tubes.

As a starting-point for this analysis we must be in possession of an exact knowledge of the various movements which occur in the bronchial tree. For practical purposes we may divide these into *a*, those in which the bronchial musculature is not actively implicated, and *b*, those in which it may be supposed to play an active part.

The movements of the first type may be briefly mentioned, only to be dismissed as not coming into the field of our discussion. Here belong the oscillations and indentations of the bronchial tubes which arise from transmitted pulsations of the heart, aorta and pulmonary arteries, and which have been viewed directly through the laryngoscope (388) and bronchoscope (194). Transmitted movements from the esophagus, caused by swallowing, are of the same order. Here may also be mentioned the deformatory bendings which appear in the bronchi in forced expiration as a result of pressure from the unusually high diaphragm domes (281). The possible changes in the angles occurring where bronchi or their subdivisions branch may also be touched upon at this point. These angles have been said by some workers to increase during inspiration and decrease correspondingly during expiration (217, 312, 313, 389, 439, 468), and to show very little or no change by others (48, 49, 280, 281, 399). Since, even if these angle changes do occur, it is impossible to see how the bronchial muscle would be actively involved in their production, we need do no more here than cite references (280, 281) where their possible relation to respiration is considered.

Certain movements of the bronchi correlated with length changes may here be alluded to. Because the thorax does not expand equally in all directions, but in certain limited directions only (217), it follows that the expanding lung must make internal adjustments in order to take full

and efficient advantage of the new space provided, and that these alterations must occur in the reverse direction in the deflation process. One such periodic change of the bronchi is said to be a torsion or twisting (440), (468—in deep breathing only). Since it has not been shown, nor does it seem probable, that the bronchial musculature is actively engaged in either phase of this movement, it may be dismissed from further consideration. Another such lung mass adjustment which is particularly well marked at the lung roots is the rhythmic shifting of the main bronchi and their branches—downward and forward in inspiration, and upward and backward in expiration. This is so concerned with the respiratory length variations of the bronchi as to warrant its consideration in the special discussion of these. The possible relation of coughing to the bronchial muscle system will also be touched upon later.

The alterations of the tree in which the bronchial musculature may be assumed to take some part are mainly concerned with respiratory movements of the lungs, and are of two types: 1, length changes of the trachea and various bronchi, bronchioli and terminal branches, and 2, width or caliber changes of these tubes. In addition to these respiratory form-changes in the bronchi may be mentioned 3, a progressional, wave-like motion resembling peristalsis, which has been observed by x-rays in bronchi filled with semi-fluid, radio-opaque substances, and which is looked upon as for the purpose of expelling masses of exudate.

Respiratory length changes. We shall now consider the movements of the bronchi which occur in respiration, and the possible part played by the bronchial muscle in their production.

It has, one would suppose, always been realized by those who gave the matter a thought, that there must be an increase in the length of the bronchi during the process of lung inflation. How could the lung mass extend its boundaries without a compensatory lengthening of the bronchial framework? Similarly, it must have been evident that in deflation there occurs a shortening of these tubes. Reisseisen, in 1822, recognized these movements and studied their structural basis in the bronchi, and since that time many workers have convinced themselves by deduction, or by various experimental procedures, that they are necessarily part of the respiratory process; indeed, hardly any doubts have been cast upon their existence (329), and these were mainly with reference to the larger tubes.

For the trachea, the majority of workers have held that it elongates during inspiration, as shown, for instance, by the descent of the bifurcation (115, 217, 281, 388, 399, 468), and nearly all investigators find

that the bronchi elongate during the same phase (171, 172, 173, 174, 280, 281, 389). Although some of these conclusions were based upon evidence obtained by technique that was not free from objection, as the making of bronchial casts by pouring in hot metal or other substances, the newer methods in vogue since the advent of the x-ray and the use of radio-opaque substances for the clearer delineation of the bronchial tree (105, 122, 150, 155, 158, 179, 193, 397, 407, 438, 466, 467, 468, 476) have served to confirm the earlier findings in this respect. That corresponding expiratory shortening of these tubes takes place is, of course, self-evident. The direct observations of these main tubes with the bronchoscope (194) show that these movements undoubtedly occur.

If the lung, for purposes of illustration, be considered as a spherical elastic sac, whose extensible bronchi, radiating from the center, are affixed uniformly to its inner surface, it is easy to see that its inflation would be equal in all directions, and would be attended by an elongation of the bronchi without change in their direction. Similarly, in the deflation of this spherical lung, there would be an equivalent shortening of the radial bronchi. Just as the volume and surface of the sphere can be enlarged only as the radii are lengthened, so is it obvious that bronchial length change is as much a part of inflation and deflation of the lung as are the increase and decrease of its volume and surface area (489).

Although the lung is far from being spherical in shape, and its internal structure is much more complicated than that of the simple sphere, its bronchi behave much as do those in the illustration, elongating, without marked, if any, change in their direction (281), as a concomitant of the inflation process, and shortening compensatorily during deflation. In this way all of the respiratory tissue in the interior of the lung mass is enabled to expand and retract. If this process be analyzed (281) it will be found that, in inspiration, the lung mass, as it were, opens up, the bronchial subdivisions which come off the same stem moving apart from one another because of the elongation of the parent stem between their points of origin, the tertiary branches from these being similarly affected. All bronchi act in the same way in this respect, and we find those supplying the apical regions, and those pointing toward the costal, mediastinal and diaphragmatic surfaces, all lengthening during the inflation process, and shortening during that of deflation.

These movements are thus an integral part of lung ventilation, and their indissociability from this is immediately realized if we but try to imagine what would be the result if the bronchial tree were rigid; if its parts were inextensible and inelastic. The lung tissue, fettered by such

a skeleton, would be absolutely incapable of expanding, save for a very thin layer of subpleural tissue, and the amount of ventilation permitted under these circumstances would be infinitesimal. An elastic bronchial system, too, is just as essential for the evacuation of the lung as for the filling of it.

The structure of the bronchial tree is admirably adapted to this length change (280); the interrupted cartilage armature, the intercartilage spaces filled with an extensible fibro-elastic tissue, the loosely-attached mucosa, and especially the "elastic membrane" already described —all contribute to build up a specialized wall which insures an even lengthening of the tubes in inspiration and a forcible, orderly and efficient shortening during expiration.

X-ray evidence. Interesting evidence on this point is yielded by roentgenographic studies. In the right main bronchus of a child a respiratory lengthening and shortening amounting to 2 centimeters has been reported (194). This was measured by the excursion of the shadow made by an iron casting in this tube, and represents the range between maximum inspiration and maximum expiration. It has been found (281) by comparing the x-ray shadows of the bronchial tree of full inspiration and of full expiration, (in films taken one immediately after the other from the same young human subject held in the same position), that, in the transition from the latter to the former phase there occurred an elongation of the trunk (trachea) and all of the branches of the bronchial tree. This was true of all tubes capable of throwing an x-ray shadow. There was, of course, a peripheral shell of lung tissue which could not be studied, with reference to its possible air-tube length changes, by this method. Similarly, in the reverse respiratory act, there was a shortening of all of these parts of the tree. Jackson (194) and others have noted by direct observation through the bronchoscope an inspiratory lengthening and an expiratory shortening of such tubes as were visible. In ordinary quiet respiration these changes would, of course, be much less extensive. Keith (217) has pointed out that under these circumstances most of the lung tissue is quiescent, and he thinks that the parts lying against moving walls are more easily ventilated than are those otherwise situated.

Functional adjustments. The adjustment transmigrations of the bronchi already mentioned may now be discussed. They are related not only to the peculiar shape of the lung, and the expansile compartment containing it, but particularly to the varying movability of the different parts of the latter. The thorax does not expand equally in all di-

rections—indeed some parts of the wall move out only slightly, or not at all. Thus the new space gained in inspiration is mainly inferior, due to the action of the diaphragm, and antero-lateral, through rib and sternum elevation, with a very little mediastinal space accession, in full inspiration, brought about by the elongation and thinning of the mediastinal contents (217, 281). There is no expansile movement of the thoracic wall at the posterior part of the apex, or in the postero-medial region (217).

How, then, does the lung expand in these regions, since it cannot push out its mass here? Not, as in other parts, by the projection outwards of the bronchial tips, but by the recession downwards of their basal ends. In what manner can their proximal ends recede from the immobile chest wall? Obviously only by the descent of their points of attachment to the main bronchi at the lung root. Keith (217) has shown that, through the direct action of the crura of the diaphragm, the lung roots are drawn downwards and forwards. Indeed, he states that the action of the diaphragm is felt as high as the apex, and that it "acts as a true piston." Thus the lung in inflation is drawn out and lengthened much like a concertina (281) when drawn out by hand, except that the lung, in addition, increases in breadth and thickness. Not only does this downward and forward migration of the lung mass involve a similar movement at the root (217, 468), but even at the lower end of the trachea.

This lung mass shifting is essential to the proper inflation of the aforementioned regions of the lung lying against inexpansile walls (217, 280, 281). Were it not for this adjustment, such regions could not be expanded; but, as it is, when the lower lung masses move away, the bronchi supplying these regions are lengthened. Thus, in the case of the bronchi serving the apical regions, through the downward shifting of their points of origin from the stems, they are elongated during lung inflation. Only by these adjustments can there be a full utilization of the aeration surface of the lung. It has even been suggested (280, 281) that we may find an explanation of the predisposition of the apical regions to tuberculosis in an early interference with lung root movement, and a consequent impairment of apical ventilation. The apical region, particularly its posterior part, is the commonest initial site of pulmonary tuberculosis, and it is just this part which first feels the effect, as an impairment of its ventilation, of immobilization of the lung root.

This up-and-down movement of the lung roots is easily seen in x-ray shadows (281), particularly if a metal rider be applied to the carina (399, 468). In forced respiration the excursion has been reported as 18

mm. (281), and this in a person of small size. In ordinary respiration, Weingaertner (468) found that the excursion was usually less than 1 centimeter. The up-and-down movement of the carina may be transmitted to an aluminum rod whose lower forked end rides upon it, and whose upper end emerges from the mouth through a bronchoscope (468). With such a preparation curves may be written, which show that sinking of the carina occurs in inspiration, and elevation in expiration. This carina movement is somewhat more extensive than the actual root movement (281), because it is due in part to dilatation of the bronchi, as well as to downward shifting.

Thus there is reliable evidence, with respect to all of the conducting part of the airway, that these tubes lengthen in inspiration and shorten in expiration; and it is evident that the same movements must occur in the respiratory portion of the airway if the lung tissue served by them is to be ventilated. Tendeloo (440) has pointed out that it is impossible to conceive of volume changes occurring in the lung without postulating the elastic extensibility and recoil of the smaller air tubes.

Shortening action of the muscle. We may now enquire as to any possible changes, active or passive, in the bronchial musculature during these respiratory length alterations in the airway. What is the mechanism of bronchial elongation? What the force which stretches these tubes? The intrapulmonary pressure, during inspiration, is greater than that within the pleural cavity, and hence the air rushes in, dilating the alveolar sacs. These are, of course, connected to the various parts of the bronchial tree (either opening directly into its peripheral ends or lying close to its conducting part), and it is obvious that they cannot dilate without collectively acting to extend the branches of the tree to which they are affixed. The combined swelling of the air-sacs enveloping any part of the airway could have no other action than to draw it out, since they are attached to it, and since they lie against one another so closely that the expansion of all must necessarily extend their total mass. It would seem reasonable to assume that where the distensible tissue is present in greatest amount, there we will see the greatest relative amount of extension, in lung inflation. This is why the peripheral region of the airway probably shows the greatest range of movement. We must not think, however, that the extra-pulmonary part of the airway will show no length changes because it has no distensible tissue around it; on the contrary we know that these changes occur in the trachea and main bronchi, and are to be regarded as accommodations to the descending lung, and are caused by the action of the diaphragm. The direction of

greatest extension of the respiratory tissue is, of course, that in which there is most opportunity for extension, and this coincides with the direction in which the bronchi run. Thus the movements of inflation are centered along definite axial lines marked out by the bronchi.

The bronchial muscle can hardly be regarded as playing an active rôle in the lengthening of the air-ducts; it is merely passive during this phase, its meshes opening out during elongation (313), and offering no impediment to this process.

Let us now examine the mechanism of expiratory recoil in the air-ducts. Shortening of these would seem to be referable to two forces; one is the recoil of the stretched, longitudinal fibers of elastic tissue, and the other is the contraction of the bronchial musculature. Inferentially, one would say that in the conducting part of the airway the elastic-tissue factor is the more potent. This action has recently been analyzed (280, 281), and the function of the elastic membrane set forth. In this part of the airway the principal result of the contraction of the muscle is to constrict the lumen; however the fasciculi are not strictly circular, but course somewhat obliquely, forming a network, and, with the tubes stretched to begin with, contraction of the muscle has here a weak shortening effect, the meshes of the net becoming narrower. The tubes shorten with a steady, even motion, with no bending or collapse, which would entrap air and obstruct the outflow (280).

In the respiratory part of the airway, too, the recoil of the network of elastic fibers would shorten, as well as narrow, the lumen. In this mural network muscle, as we have seen, forms a substantial element, and its contraction would also shorten and constrict the lumen. The shortening action of the muscle is probably relatively stronger in these passages than in the conducting part, because the fasciculi run more obliquely.

In all the passages, the steadiness and smoothness of the recoil process suggests, perhaps, that the mechanism may not be merely the rebound of an elastic tube following the removal of an extending force, but a movement in which muscular activity, coördinated under nervous influence, is a factor. Miller (313) and others regard the bronchial muscle as playing an active rôle in expiration. A further consideration of this possibility will be taken up in the discussion of caliber changes in the airway.

In the trachea, of course, muscle contraction would be of little avail in shortening the tube, since it would affect only the flaccid posterior wall, and this mainly by a narrowing action.

Respiratory caliber changes. The bronchial musculature has been

pictured as a network whose fasciculi course in a direction mainly transverse. Its structure is obviously for the primary purpose of narrowing the lumen, but it is almost equally plain, as has been pointed out, that with the tubes extended and dilated to begin with the contraction of the muscle would shorten as well as narrow them. This is manifestly the only way the muscle-system can act, because of its anatomical arrangement (189, 326)—a conclusion arrived at by Reisseisen (364) from his dissections.

The disposition of the other constituents of the airway wall permits just as readily of changes in width as those of length. The flexible cartilage segments—never forming complete rings; the fibro-elastic principal coat, rich in circular elastic fibers, and the redundant mucosa, easily thrown by contraction into longitudinal folds, which tend to smooth out in dilatation—all combine to form a tube eminently adapted to variation in caliber.

Although the above-mentioned changes in length and width are physiologically inseparable, yet for analytical purposes we shall now consider variations in width alone, and shall review the evidence for these changes as they may occur at various levels of the two main divisions of the airway. We shall enquire as to their possible relation to respiration, and as to the rôle, if any, played by the bronchial muscle-system in their production; and, later on, we shall also glance at the part taken by this motor-mechanism in the expulsion from the various parts of the bronchial tree of exudates, or of foreign materials which have been injected into them.

First, let us attempt to review the evidence respecting these caliber changes in respiratory movements. Various techniques have been used, but so far none of them has yielded entirely satisfactory results, and many have been worthless or even misleading. In the last group may be placed the cast methods (3, 313, 336, 337 et al.) which have been used in an attempt to obtain information as to the size of the lumen in various parts of the bronchial tree by pouring melted substances, often very hot, into the trachea or large bronchus (see 468 for bibliography). These substances subsequently harden and the preparation is examined usually after removal of the lung tissue, by corrosion or digestion. A favorite material has been hot Wood's metal. It is obvious that a heavy molten mass will cause distortion of the tubes, not only from its own weight (41, 49), and the erosive action of its latent heat, but also from the steam which is generated while it is being poured in. Melted paraffin has the same objections in lesser degree. Then, too, these methods have to be used on

dead animals, so that true physiological conditions cannot be registered, nor can accurate comparisons of physiological stages be made. Undoubtedly this is one of the reasons why the results of various workers respecting caliber changes have been so conflicting, and why the condition of our present knowledge is so chaotic. The author would brush aside all these results from casts as worthless in the solution of the problem of bronchial caliber changes in respiration.

X-ray evidence. The radioscopic evidence is, on the whole, inconclusive. The shadows of normal untreated bronchi are too indefinite to be studied for width changes on the fluorescent screen, and their outlines are usually very indistinct even in the best plates or films. However, it was possible (281), by the selection of a favorable subject and the employment of suitable technique to make x-ray films in which the bronchial contours could be made out in limited areas. By taking two such films from the same subject, so as to show the contrasting phases of full expiration and full inspiration (one taken immediately after the other with the subject held in exactly the same position), and by comparing them in view-boxes, the large bronchi in the region of the root were seen to be considerably wider in full inspiration than they were in full expiration. This was clearer on the left side than on the right. Of course, even under these favorable circumstances, most of the bronchial contours were too indefinite to permit of width changes being studied. It will be noted, too, that this was a comparison of the condition of the tubes at the *end* of a *forced* expiration with that at the *end* of a *forced* inspiration, and the results cannot be used in the elucidation of intermediate events in the respiratory cycle. In this case the tracheal shadows at these extremes of the respiratory cycle were the same.

It has been possible to make the bronchial outlines clearer by the insufflation, into the airway, of radio-opaque powders (406, 438, 468, 480, et al.), and, in recent times, by the instillation of iodized oil (122, 194, et al.), an operation which has now been performed upon almost countless cases and regarding which a vast literature has already accumulated.

It is probable that, with foreign material in the bronchi, the physiological action of the walls will be somewhat abnormal, as far as respiratory movements go: indeed, this is certain where the injected or insufflated material fills the lumen, as is usual in the smaller tubes. This objection is probably not valid when the walls of the larger tubes are dusted with only enough powder to adhere to them (468), or are sprayed with iodized oil. Then, too, the movements are difficult to follow on the screen.

If the breath is held in any phase of the respiratory cycle, one cannot be sure that there is presented an accurate picture of the caliber of the tube during action.

We are just at the outset of experience with such methods, however, and it is more than possible that the future will provide us with means of mapping accurately and without physiological impairment not only isolated part, but the entire bronchial tree. We may even be able to study all the bronchial movements which occur during respiration with the fluorescent screen, and permanently register all phases of these on x-ray films or plates. Although up to the present these procedures have given us much help in the general study of the normal and abnormal bronchial tree, particularly by the stereoscopic method of recording, they have yielded little of value as to the respiratory width changes.

Bronchoscopic evidence. The bronchoscope has given very positive results respecting the respiratory movements of the bronchi. The objection (48, 49) that these may be altered by the presence of the stiff tube has probably little or no weight. True, the field under observation at any one time is very limited, but considered as a whole, the territory of the bronchial tree which can by this method be progressively covered is very considerable. In the hands of Fletcher Ingals (191) and Chevalier Jackson (193, 194) this instrument has been particularly helpful, and the author would go so far as to say that the only incontrovertible evidence respecting bronchial width changes in their relation to respiratory movements has been gained through direct observation of the moving parts through the bronchoscope. It is very definitely established that, in all of the respiratory tract which can be thus inspected—the trachea, the main bronchi and their larger branches—there is an inspiratory dilatation and an expiratory narrowing. The author has observed very marked movements of this type in the dog. The inspiratory dilatation can be seen to be associated with elongation, and the expiratory narrowing with shortening (194); in fact this association is inseparable, and these movements are indissociable from respiration.

Weingaertner (468) points out, in confirmation of the observations of v. Schrötter and Brünings, that in quiet respiration these movements, in the trachea and large bronchi of the adult, are very slight. Ingals (191), by bronchoscopy, found no tracheal lumen change in the adult, but marked movement in the child.

The finer tubes. Unfortunately, the smaller passages of the airway cannot be inspected with the bronchoscope, and neither x-rays nor any other method has yielded us anything of value regarding respiratory

width changes here.* However, we know that the general structure of the smaller bronchi and bronchioli is fundamentally the same as that of the bronchi of larger caliber, and indeed that the muscle, particularly in the smaller bronchioles, is relatively more dense than it is in the larger tubes. Thus we are justified in concluding that the smaller branches of the conducting part of the airway behave in respiration similarly to the larger ones (468); indeed we may look for a relatively more forcible action of the muscle here. The findings of Schulze and Jordan (393) in anaphylaxis indicate that the muscle of the secondary and tertiary bronchi may so constrict the tubes as to obliterate the lumen.

In the respiratory bronchioles and the alveolar ducts we lack experimental evidence respecting caliber changes; however, it may be inferred that wherever smooth muscle is present in the form in which it is found in the bronchial tree, the tube containing it must be capable of caliber variations. Indeed, it is the consensus of opinion that we may expect to find relatively greater respiratory caliber fluctuations here than in the conducting part. We know that the wall of these passageways is a contractile net whose meshes lead into dilatable air-chambers, and we have concluded that the tubes must elongate in lung inflation and shorten in deflation. It seems just as likely, too, that they expand and contract, respectively, during these phases. Just as in the larger tubes, so here, lengthening and dilatation go hand in hand, and so do shortening and narrowing. The changes in the inflation process involve a widening of the meshes of the contractile net. Each mesh is a sphincter leading into either a branch of the alveolar duct or into an alveolar sac or alveolus—in any case into a distensible air chamber. Thus we may picture these sphincters as air-mouths, widening during inspiration and narrowing during expiration, and in this way facilitating the ingress and egress of air to and from the respiratory chambers. The inflation of the alveolar sacs is dependent upon the simultaneous lengthening and expansion of the tube into which they open. The action here is like that of the lung of the frog, as we might expect it would be, from the likeness in fundamental structure.

Constricting action of the muscle. If we ask ourselves what is the cause of air-duct dilatation, we find a ready answer in the inspiratory plus pressure existing in the airway as compared with the pleural cavity. The purely conducting tubes are dilated because the air-pressure within them during inspiration is greater than that of the surrounding lung substance. In the respiratory passageways, the fact that the lumen is in such free communication with the annexed respiratory chambers

*See appendix.

that we might almost consider them as making up a single compartment leads us to assume that the meshes of the net-wall of the tubes here would be pulled open by the tension of the walls of the dilating chambers of which these meshes are the mouths. The synchronism of dilatation and elongation is explained by their being produced by the same cause.

The muscle, of course, in this dilatation process must be considered as passive, merely yielding, with the other constituents of the air-tube wall, to the intraluminal pressure. It seems likely, however, that the muscle is, during this phase, under the influence of nerves, the broncho-dilators, whose action at this juncture is to relax it, and permit of its fibers being extended without resistance. The circular elastic fibers, of course, are simultaneously put on the stretch.

Nothing seems to be known regarding the details of this dilatation process; as whether or not it goes on simultaneously throughout the entire extent of the bronchial tree, or progresses as a wave which travels from the larynx to the respiratory chambers. If such a wave did occur we would want to know the details about its curve, as for instance the point in the inspiratory phase at which it was highest. Would such a curve be like that tracing out the negative pressure change of the pleural cavity, in inspiration?

The withdrawal of the force making for the expansion of the lung leaves the stretched elastic tissue system of the airway free to contract. This recoil process, while shortening the tubes, at the same time narrows them through the action of the circularly disposed elastic tissue fibers.

The question arises, is this elastic tissue system sufficient of itself to empty the lung satisfactorily, or does it require assistance from the bronchial musculature in this process; indeed, in deflation, is the primary rôle played by the muscle? Is the muscle capable of constricting the tubes beyond the point when the elastic tissue ceases to act? It has frequently been pointed out that if the bronchial musculature has any function at all in ordinary respiration it must show it in the expiratory phase (217). Its contraction would undoubtedly narrow the tubes and also shorten them. The constricting effect of the muscle would be more apparent in the conducting part than in the respiratory passages, and relatively most evident in the smaller bronchioles. That the muscle-system acts in collaboration with the elastic tissue system in the expiratory shortening and constriction of the tubes is strongly suggested. The observations of Ingals (191) on dogs showed that although the lumen changes were present after section of the vagus, the contraction was not so marked, thus indicating that the muscles, normally under vagus con-

trol, assist the elastic tissue in narrowing the bronchial lumen. There are interesting problems to be worked out regarding the details of this constriction process. Are we to picture a simultaneous narrowing (363) over the whole system, or an expiratory wave travelling from the air-way-terminals to the larynx? It seems obvious that such a wave would best serve in emptying the lung, for too early an expiratory contraction of the larger bronchi would offer an impediment to the outflow of air. And, as in the case of dilatation, we may ask what is the shape of the curve of constriction? Further research may clear up these points.

The dynamic bronchus. Thus, on the whole, the available evidence and such deductions as can be made relative to the respiratory movements of the bronchi point indubitably to an inspiratory dilatation with lengthening, and to an expiratory constriction with shortening.

It would seem hardly likely that the caliber adjustments to the respiratory phases should be other than these. Is it not reasonable to suppose that, at the time when air is entering, the tubes should be at their widest, so that the air may be most freely admitted (98)? These considerations, particularly the bronchoscopic evidence, make us discount the view that there is an inspiratory constriction of the tubes (313, 317), (trachea and large extra-pulmonary bronchi only, 329); or that they show no change in respiration (48, 49); or that, while showing inspiratory dilatation in children the tubes do not change their caliber in the adult (191). We feel that these discrepant findings are due to faulty methods or abnormal subjects studied. It must be admitted, however, that the extent of the caliber change is probably in direct relation to the extent and vigor of the respiratory act, and that in quiet breathing it may be very slight, particularly in the conducting part (491).

The reason that the tubes can become narrower at the same time that they shorten, and wider while they are lengthening, is because the fasciculi of muscle are contractile and extensible, and not inextensible as in the case of the wire of a coiled spring. The lumen of the latter, when it is drawn out, becomes narrowed and, contrariwise, when the spring contracts, the lumen is widened. The behavior of the air-tube is quite different for, at the same time that it is being elongated it is becoming wider, due to the relaxation and stretching of the walls, and, on the other hand, it narrows simultaneously with its shortening, from the contraction of the walls. The likening of the action of the bronchial muscles to that of a lazy tongs (313) is not altogether appropriate for the same reason, namely, that the lazy tongs becomes narrower as it elongates, while the bronchus becomes wider.

This is no mere academic question, and failure to solve it correctly has led to erroneous conceptions of the physiology of the bronchial tree. The important point to emphasize here is that, because the bronchial tree widens as it elongates, its *capacity increases*. Were it to be merely elongated like a rubber tube the volume of its contents would remain constant. There is thus an inspiratory increase in the volume of the air in the bronchial tree, and this increase is mainly in the respiratory division—in the "bellows part" of Keith. This increase in bronchial capacity is doubtless in direct proportion to the force and depth of breathing, although it is impossible to conceive of any respiration at all which does not involve this change in some degree.

It is also apparent that in expiration, because the tubes of the bronchial tree become narrower at the same time that they are shortening, the *capacity decreases*. As before, the greatest change is in the respiratory part of the airway. Thus the bronchial tree can obviously have no "normal" or "constant" capacity, for this varies with the phase of the respiratory cycle, and with the depth of breathing. Its constancy is one of change.

The dead space. By this time it will be strongly suspected that what we have been describing as the volume of the lumen of the bronchial tree is no other than the "dead space" of certain of the physiologists, but presented in an anatomical manner. It is rather mystifying to attempt to grasp the various conceptions of the "dead space," arrived at, as they have been, by so many different methods, and although some workers present this space as a physiological entity, different from the anatomical dead space, it seems quite certain that the idea in the minds of a number of physiologists coincides with what we have described as "bronchial capacity."

It is obvious that the anatomical dead space, as we have presented it, must be a variable quantity. Yet certain investigators (Pearce and Hoover—483) have solemnly averred that the dead space is a "relatively fixed anatomical and physiological entity," and have invoked the aid of the binomial formula to prove it so. They even despair of settling the question of the variability of the dead space otherwise, for they say "If such a mathematical formula, theoretically sound and utilizing absolutely normal data, fails to give correct values for the composition of the alveolar air or for the dead space, we are at a loss to find a basis to solve the problem." Others (Krogh and Lindhard—481) state that its volume is not altered in exercise; or (482) at least not more than 100 ccm.

This conception of fixed bronchial capacity seems to be at the root of

much of the difficulty in the interpretation of the significance of the dead space. We feel that not one of the workers who regarded this space as of fixed volume could have gained such an erroneous notion if he had dissected the bronchi carefully, or studied histological sections of them, for then the manifest provision for width and length changes would have forced a different conclusion. How refreshing to find physiologists like Yandell Henderson and his co-workers (484) who recognize that "the respiratory dead space, like other cavities having non-striated muscle fibers in their walls, is subject to considerable active variations of a more or less rhythmic character!"

And there were others who had, before this, looked upon the capacity of the dead space as variable. Siebeck (486, quoted by 484), while recognizing that it could be altered, went astray in thinking that depth of breathing was not a factor in this change. Douglas and Haldane (98) grasped the essential conception that it was due to respiratory movement that the dead space was altered, and they found that it could be increased as much as fourfold after muscular exercise. This enlargement, they said, was brought about through active bronchial dilatation, and was for the purpose of facilitating ingress of air during inspiration. Henderson and his co-workers (484) found that the increase in dead space volume could be even sixfold or more, and depended on depth of breathing regardless of whether the subject was exercising or not; but they attributed it to a passive dilatation of the passages and not to active broncho-dilatation—a view in which Haldane later (485) concurred. They found also that there were rhythmic variations in the size of the dead space even with the chest in a position of uniform inflation (see 490).

It is true that some of these workers (484, 485) have regarded the "atrium" of Miller—a structure of whose existence we are not certain—as the essentially distensible part of the dead space. They are undoubtedly correct in locating the greatest variability in volume toward the periphery of the system, but it must not be overlooked that any respiration, however slight, involves some bronchial lengthening.

It is apparent that many workers have had in mind a conception which might be likened to the inflation of a rubber balloon through a rigid pipe, say a piece of glass tubing. The tubing would represent the rigid, inextensible bronchial tree; the balloon, the alveolar tissue. This presupposes two hypotheses, both of which are untenable. The rigid tree, as we have shown, would not work at all; in fact it would utterly interdict lung action. The inflated alveolar tissue would call for the stretching of the walls of the alveoli—a conception which, except in very slight

degree, would be equally unworkable, for (to mention only one disadvantage) it is apparent that stretching of the alveolar walls would be likely to narrow the capillaries and thus impede the pulmonary circulation in inspiration; on the contrary it is accelerated.

Thus the *ignis fatuus* of a constant dead space and distensible alveolar walls gives place to the substantial fact of a variable dead space and alveoli whose walls undergo little or no stretching, but merely become shallower and wider at the mouth, when the tube, into which they open, dilates. Keith (217) has given us a diagrammatic conception of this process. Nor is this idea new—in fact the germs of it are to be found in the work of the anatomist, Reisseisen, done during the period of the Napoleonic wars, and other anatomists, like Keith, have amplified it (217).

We feel that this conception of the "dead space" should arrest its obsequies; or perhaps it would be more appropriate to say it should lead to the exhumation of its real meaning. It is most essential to regard this space as variable, since its variations are indispensable in lung function. The respiratory variations in bronchial capacity are plainly in the interest of thorough pulmonary ventilation; the more fully contracted the air tubes are at the end of expiration, the less alveolar air there is to be sucked back into the alveoli at the next inspiration, before the new air gains access to the respiratory epithelium.

But the important question here is: what rôle, if any, does the bronchial musculature play in the control of bronchial capacity? Admittedly, it is in a position to exert a strong influence on this. Its contraction, as we have seen, could have no other effect than to diminish the intrabronchial space—to drive the air out. Thus, if its contraction is properly coördinated, it is able to facilitate expiration. In inspiration too, while taking no active part, it could hamper the process if it did not relax and thus permit the tubes to be dilated and lengthened. Indeed it seems not too much to infer that the bronchial musculature is an essential in respiration, for it is involved in both the opening and the closing of the pulmonary bellows. The muscle is undoubtedly under nervous control; indeed an elaborate system of nerves governs its action, which will be discussed in a special section of this paper. To rule it out as a respiratory asset would be to ignore a neuro-muscular system which one might reasonably regard as designed specially to function in expiration, and to leave the pulmonary factor in this process entirely in the hands of the stretched elastic fabric, which is quite beyond nervous control, and which acts only passively.

We might ask whether or not the various regions of the bronchial musculature are able to act individually; if the trachea, main bronchi, etc., are capable of more or less independent action, in respiratory movements. Then, too, it would be interesting to know if there are important changes in the dead space in respiratory diseases, such as pneumonia. Henderson (484) suggests that this is so in asthma.

Accessory respiratory movements. Somewhat similar caliber changes have been described occurring under conditions other than in the course of ordinary breathing. Thus Brünings (48, 49) made direct observations of the trachea and bronchi through a tube in a tracheal fistula, and reports that a momentary narrowing of the trachea and visible bronchi occurs when the glottis suddenly springs open under strong expiratory pressure; or when, with an open glottis, a sudden expiratory effort is made, as in coughing.

Weingaertner (468) reports a widening of the tubes under the maneuver of Valsalva (expiratory effort with closed glottis), and a narrowing of the trachea and upper bronchi with Müller's maneuver (inspiratory effort with closed glottis). He states that in children the trachea is so flexible that it may be completely closed in forcible expiration.

Henderson (484) reports some facts which "suggest that in a 'close and stuffy' room the bronchi, etc., are constricted, and that a distinct chill constricts them further, while pleasantly cool air, on the contrary, induces broncho-dilatation."

The details of muscle involvement in these movements, and in others, such as phonation, and sneezing, are not known.

Other suggestions have been made as to ways and means by which the bronchial muscle may contribute to respiration. For instance, it has been hinted that the muscle rings of the airway terminals, which act as sphincters for the respiratory chambers, may have something to do with the regulation of the pressure of the air within the alveoli (217, 313), though just how this is done, if at all, is not clear. The muscle of the bronchioles, too, has been thought by some (217, 426) to act like that of the arterioles in the case of the blood current, that is, to control the air-flow to the various regions of the lung, constricting in one region and opening out in another, under the supervision of the nervous system, to the end that the air may be directed into this or that portion of the lung substance. Such a supposition is entirely speculative and there is, as yet, no evidence in support of it. Thus Krogh and Lindhard (482) state: "no evidence has been obtained of any unequal ventilation of different lobes of the lungs." It has been suggested, too (217) that in-

directly the pulmonary blood flow may be similarly influenced, and that thus the bronchial musculature may, in a measure, take the place of the vaso-motor mechanism.

Expulsive and protective movements. Because of the ease with which foreign bodies, from very small particles to quite large objects, can gain access to the respiratory tract, and also because in inflammation of this tract exudates accumulate within it, there arises the necessity for means of freeing the tract from such obstructions and irritants. To this end we find three mechanisms developed: the cough reflex, the action of cilia, and a wave motion said to resemble peristalsis (363), all of which often work together. According to Gunn (487) the cough reflex functions in the upper airway and the cilia as far down as the finer bronchioles, while peristaltoid movements evacuate the entire tract even including the airway terminals. Thus the fields manifesting these activities overlap somewhat, the upper airway having all three, the intermediate part two, while the terminals, if Gunn's view be correct, would have only one mechanism for evacuation, viz., that of wave motion.

Peristaltoid movements have been described in the bronchi by a number of authors (53, 54, 180, 363, 390 et al.), and it may be said that they are dependent entirely upon the bronchial muscles, and that they seem to be called strongly into play only under abnormal conditions, viz., in the ejection of masses of thick exudate or other foreign material from the lumen. This function is regarded as very important, indeed indispensable, under these conditions. It has been described in the human subject (53, 363), as well as in animals, such as the dog (53, 54) and the goose (363), and its study has been carried on mainly by x-rays, following the injection of an opaque mass, as bismuth paste, into the bronchial tree. That the structure of the bronchial musculature, through its resemblance to that of other tubes which show peristalsis, is adapted to this form of movement, is claimed by Reinberg (363).

The "peristaltic" wave is said to resemble that of the digestive tract (363), and its length may reach to ten centimeters. The speed of masses so propelled is too rapid to be accounted for by ciliary action (53, 54, 363). Bullowa and Gottlieb describe it as "bellows-like," and Reinberg has found it so marked in the trachea as to suggest the designation "tracheal vomiting." The alternate contraction and dilatation which occur at any one point of the tube are independent of coughing, swallowing or respiration (53, 54, 363). These movements could not be confused with those of circulatory or respiratory origin (363).

Bronchial "peristalsis" is a reflex movement dependent upon the

integrity of the mucosa, and presumably of the sensory endings found there (245, 246, 247, 248, 249), and does not occur if the mucosa is destroyed. Hence it is under the control of the nervous system (54). Bullowa and Gottlieb look upon this phenomenon as an emergency function of the muscle, distinct from caliber control, which they think is its usual duty; and they raise the question as to whether or not there may be two distinct innervation mechanisms for the regulation of these two activities. This movement has not been definitely demonstrated in the respiratory part of the airway, although Gunn infers that it may be called into operation here.

It seems likely that the rhythmic variations of the dead space described by Henderson (484) were peristaltic waves, and also that the slight rhythmic variations of bronchial tonus described by Einthoven (109) in dogs were of the same order. One may ask: is the bronchial tree always in a condition of slight peristaltoid movement? Is it, like a perpetually moving conveyor, an "escalator" if you will, always manifesting this progressional activity, whether or not there is a burden to be carried? And is this movement of value in respiration—in eliminating gaseous waste?

Bronchial peristaltoid movement is reported as being influenced by pathological conditions (363) and as probably more active in gangrene and abscess of the lung. It is less active in bronchiectasis, which is accompanied by a degeneration of the muscle. Opium and its derivatives inhibit it.

This action of the bronchial muscles is not at all surprising in view of the fact that they have a common derivation with those of the intestinal tract (178, 216, 236, 451), although they have been described as arising from entoderm (226, 228, 230). Their motor innervation, by way of the vagus, and the presence among their fasciculi of Remak's ganglion, corresponding, perhaps, to the ganglia of Auerbach and Meissner, is in harmony with the idea of a peristaltic action in them.

In this connection, the beautiful experiments of Margaret R. Lewis (257) may be mentioned. In tissue culture preparations from young chick embryos of 10 to 16 days' incubation, this observer noted the occurrence of spontaneous rhythmical contractions in the respiratory tubes. The arrangement of the muscle was such that contraction caused not only constriction, but also shortening. Waves, passing along the entire tube, were noted. Since nerve fibers were rarely found, it was concluded that this type of contraction was a function of the muscle itself.

Before concluding this section it will be well to mention that contraction of the bronchial musculature as a result of inhalation of irritating substances (as vapors) has been described (186), and has been regarded as protective in function.

THE INNERVATION OF THE BRONCHIAL MUSCULATURE. The nervous control of the bronchial musculature is, of course, located in the autonomic system, and the evidence which has accumulated in quest of information as to just what nerve centers are connected with the muscles, and over what pathways the impulses travel, is of several kinds—physiological, anatomical and pharmacological. A full discussion of this subject cannot be entered into here, and only brief mention can be made of the more important results.

The *physiological work* has been mainly centered on the question of the presence of broncho-constrictor and broncho-dilator fibers in the vagus nerve, whose peripheral end has been stimulated and the resulting activity of the bronchial muscles noted. Workers have been divided into three groups: 1, those who have found that the vagus carried broncho-constrictor fibers (25, 28, 36, 68, 95, 109, 116, 126, 127, 139, 191, 202, 211, 222, 251, 265, 266, 271, 368, 453); 2, those who found no broncho-constrictors in the vagus (96, 379, 386, 443, 472); and 3, those who found both constrictors and dilators in the vagus (45, 94, 99, 125, 321, 378, 381).

One of the most probable causes for this conflict of views lies in the fact that different investigators used different animals. That conclusions based on the results obtained in one species are not generally applicable is shown by the work of Carlson and Luckhardt. They found that the vagus of the salamander contains only broncho-dilator fibers (268); that of the turtle, only broncho-constrictors (64); while that of the frog contains both types (63). The vagus may be in reality a vago-sympathetic trunk, and there may be spinal as well as medullary centers for the control of the bronchi, thus further complicating the results (39). It would seem that there is no doubt about there being both dilator and constrictor fibers for the bronchial muscles, but their pathway must be determined for each species used.

The central origin of these two sets of fibers has been described by Möllgaard, who (according to Larsell—248) claims that the broncho-constrictors have their origin in multipolar cells, in the dorsal nucleus of the vagus nerve, and that the dilators arise in unipolar cells in the nodose ganglion. Molhant (324) states that the motor fibers for the bronchial muscles arise in the dorsal nucleus of the vagus and that the

sensory fibers arise in the nodose ganglion (325). Head (175) found the sensory fibers from the lungs terminating in the first five thoracic ganglia.

The centers for broncho-constriction may be reflexly stimulated, the most effective reflex arising through mechanical or chemical stimulation of the nasal mucosa (1, 7, 38, 42, 46, 102, 117, 123, 124, 128, 148, 190, 237, 270, 284, 375, 381, 403, 433, 452, 454, 455). To a much less degree stimulation of other regions of the body may cause constriction of the bronchi, the motor pathway for these effects being the vagus (75, 356).

There are sensory pathways for impulses arising in the bronchial muscles as well as in the mucosa, and there seems to be confusion here as to whether it is the vagus or the sympathetic which is responsible for the carrying of these impulses. Probably both are involved, and the extent to which they act depends, no doubt, on the animal used and the type of impulse mediated. The vagus is held chiefly or solely responsible for the transfer of sensory impulses by some (249, 285, 360); while the opposite viewpoint, that the vagus is little or not at all concerned, is also maintained (80). From clinical evidence Pottenger (355) concluded that both vagus and sympathetic are active in the conduction of afferent impulses from the bronchi and lungs.

Anatomical evidence, based on dissections, nerve degenerations, and histological researches demonstrates the existence of motor nerve fibers destined for the bronchial muscles and their endings in the muscle cells; of sensory fibers arising in the muscles, and of numerous ganglia in the bronchial wall from which arise the postganglionic fibers destined for the actual muscle cells.

Schiff (385) found that the vagus supplied fibers to the bronchi and lungs, and Chase and Ranson (67) state that most of the medullated fibers present in the thoracic vagus leave the nerve in the bronchial branches. They are either sensory fibers, or the preganglionic fibers of the motor nerves. Terminations of the postganglionic fibers on the actual muscle cells have been described (26, 27, 245, 246).

Ganglion cells in large and small accumulations, from which proceed fibers toward the muscle cells and about which terminates the pericellular network from the preganglionic fibers, have been described since the time of Remak (365), after whom the ganglia system of the bronchi is named (26, 27, 50, 83, 108, 206, 214, 245, 246, 247, 248, 250, 258, 310, 347, 349, 365, 366, 394, 400, 428, 473).

The anatomical evidence, as well as the physiological, for the presence of sensory fibers from the lungs and the bronchi, has been reviewed by

Ranson (359). The chief point of interest here is whether there is some structure to mediate sensory impulses arising in the bronchial muscles. The evidence is not abundant. Larsell (248) shows figures of sensory terminations which wind about and end on the muscle cells, the "sensory muscle spindles." He says: "It does not appear impossible that the smooth muscle spindles in the bronchial musculature may exert a regulatory influence on the respiratory movements, through the action upon them of the contracting and relaxing muscle bands during inspiration and expiration." Similar muscle spindles to those described by Larsell have been found in the reptilian lung (206). Sensory endings in the mucosa have been pictured by Larsell (245, 246), and these provide another pathway for reflex stimulation of the motor nerves.

Correlation with bronchial movements. This complex nervous mechanism regulates the various bronchial movements already discussed. There is provision in it for the mediation of both broncho-constriction and dilatation, through reflexes involving the vagus and the sympathetic trunks. All the necessary nerve components for such reflexes, arising within the bronchial muscles themselves or from stimuli outside them, which might influence the tension of these muscles, are present. A correlation of the entire assortment of bronchial movements with this nervous mechanism cannot be attempted here, yet it may not be unprofitable to pass in review one or two points related to the nervous control of the bronchial muscles in respiratory movements.

Most workers who have examined the bronchial musculature have found it easier to think of it as being actively engaged in the expiratory process (263, 265, 426)—as acting to diminish the dead space—than as being merely passive in this stage, and the writer acknowledges that he is of their number. It may, then, seem not inappropriate at this juncture to hazard the following discussion in an attempt to find an explanation of the manner of working of the bronchial neuro-muscular mechanism in movements of ordinary respiration.

It has long been agreed by physiologists that the stimulus to expiration is the attainment of a certain stage of distention in the lungs; in other words, inspiration initiates impulses which, travelling over the vagus, influence the respiratory center and inhibit the further outflow of inspiratory impulses. May it not be that the same impulse that inhibits the inspiration stimulates, by means of other connections, the center in the vagus nucleus which causes the bronchial muscles to contract? Hence, when the chest wall begins its relaxation, the lung finds a muscular tube beginning to contract, and accommodating itself in size and

length to the diminishing size of the lung. Expiration, passive in the external muscles of respiration, may be looked upon as the active phase in the bronchial tree. The associated recoil of the elastic tissue of the lungs and bronchi during the expiratory phase, of course, is not overlooked. The vagus is looked upon as the motor nerve of the bronchial musculature, and many experiments support this. Although section of both vagi is said by Starling (408) sometimes to leave the respiratory pressure curve unaltered, it may lower it slightly, indicating a certain tonic influence on the muscles exerted by this nerve. Fletcher Ingals (191), too, reported that in dogs the movements of the bronchi still continued after vagus section, though apparently within a narrower range. Stimulation of the distal end of the vagus reduced the caliber of the bronchi to one-fourth of the diameter in inspiration; and he concludes that while the movements were in part passive they were also in part dependent upon the muscular contractions resulting from the action of the vagus nerve. His work seems particularly convincing since he actually viewed the moving parts through the bronchoscope. The tonus of the vagus can be brought into play by inhalation of three to four per cent of carbon dioxide.

The bronchial muscles, supplied by sensory spindles, contract until the end of a normal expiration is reached, at which time the sensory termination may be assumed to be stimulated and the impulse, travelling by way of either the vagus or the sympathetic, finally reaches the broncho-constricting center which is thus inhibited at the end of expiration by impulses arising through the expiratory act being carried to a certain stage, exactly as the inspiratory center is inhibited at the end of inspiration by impulses arising through the inspiratory act being carried to a definite degree. Thus, when the respiratory center sends out its new impulse to the inspiratory chest muscles to contract, there is within the chest a bronchial tree contracted, but with little or no tone in its muscles, ready to follow passively the next phase of inspiration.

It may be that evidence which has been presented in the past to prove that there are fibers in the vagus which are stimulated at the end of expiration to initiate a new inspiration (a theory questioned by many physiologists), points to the presence, not of inspiratory fibers in the lung, but of sensory nerve endings in the bronchial musculature which are stimulated by the contracted muscle at the end of expiration to inhibit further muscle constriction. Einthoven's work (110, 111) with the string galvanometer on the inspiring and expiring lung has been used in the past to support the theory of inspiratory as well as expiratory

fibers in the lung. He says, "The latter (meaning the inspiratory) act more weakly, are sooner tired, and are killed more easily by injuries than are the former (referring to the expiratory) so that the two kinds of fibers can be separated from each other as to their action." This finding would agree equally well with the idea that the action current which was registered at the end of expiration was due to the sensory impulses from the muscles of the bronchi, rather than from the collapsed alveoli of the lung. Since he finds such a striking difference in the action of the two sets of fibers, the theory is even more strengthened that it is sensory impulses from the bronchi that are being registered. Sensory impulses from distended and from collapsed alveoli are apt to be more nearly uniform.

Pharmacological results. A large mass of data obtained by observations on the effects of various drug-stuffs on the bronchial musculature has been accumulated, and attempts have been made to elucidate the problem of nerve control of these muscles by a study of these results. It is not denied that the methods have been far from ideal, such as studying the action of isolated strips of bronchial muscle drenched with unfamiliar drug-stuffs: and the eligibility of their use on physiological problems has been questioned (363). It is felt however that a discussion of the innervation of the bronchial musculature would not be complete without at least a brief survey of the pharmacologists' contributions.

Of such drug-stuffs there are two classes, the constrictors and the dilators. These may be further subdivided according to the structure upon which they act: thus, of the broncho-constrictors, there are those which: 1, stimulate the vagus endings in the muscles; 2, act on the muscle directly; 3, inhibit the sympathetic; 4, are central in action; 5, have an unknown site of action.

The drugs belonging to the first class, namely, those which stimulate the vagus endings, include the following: muscarine (94, 153, 205, 278, 279, 339, 356, 449, 450); arecoline (198, 200, 449, 450); physostigmine (94, 279, 339, 356), although this drug has been said to be inactive on isolated bronchi of the cow (450) and shown to increase the output of the adrenals (421), which action might be inclined to overshadow its constricting effect; digitalin, (94, 465); neurin (94); aspidiospermin (94), although it has been said to be inactive (78); tetramethylammonium chloride (66); vanadium (195); cholin ethyl ether, and cholin esters of acetic, nitrous and nitric acids (89).

The second class, those which act directly on the muscle cell, comprise barium chloride (94, 279, 350, 351, 465); vanadium salts (195); bromine

(79, 94, 434, 435), but in large quantities gave relaxation (279); ergotoxin (87, 144), although it is said to be inactive on bronchial muscle (350, 351) and on tracheal muscle (146), and is claimed to be first a dilator due to its stimulation of the adrenals, and then a constrictor through action on the muscle cell (196, 197, 198, 200); thebaine (198, 199, 200, 272), but claimed to have little effect (279); peptone (17, 163, 339, 465), but claimed to be inactive (450); veratrin (94), although claimed to be inactive (450); histamine (90, 163, 164, 200, 279, 283), although claimed to be inactive (146, 450); quinine, (197); atropine, only after it has paralysed the vagus endings (197); morphine (272), although said to have little effect (279); pantopon and nicotine (199), but said to be dilators, not constrictors (279); monacetylmorphine, neopin, dionin, peronine, (199); codeine (199, 272, 283); heroine (199), but constricting effect has been denied (279).

In the third class—those drugs which constrict the bronchi through inhibition of the sympathetic endings—we have ergotoxine (279).

To the fourth class—those which are central in origin—belongs carbon dioxide (94, 109, 449, 450).

The drugs belonging to the fifth class—those which constrict the bronchi, but whose site of activity is unknown or not stated—are as follows: curare (144); sodium carbonate and bicarbonate (279); acids (279), although the hydrogen ion is said to cause relaxation (450); antipyrine (279); ergotoxine (356), nicotine and imid (350, 351); OH ions (450), but in strong concentrations cause relaxation (279); chlorine, relaxes the muscle in low, but contracts it vigorously when present in high concentrations (22); since this constriction is unrelieved by morphine (21) or by epinephrin (19) or by calcium, quinine or atropine (20) it seems probable that chlorine exerts its action directly upon the muscle. The constriction caused by chlorine is relieved by injections of atropine (79), and by inhalation of atropine (434, 435). Pituitary extract caused constriction, but its activity could be prevented by atropine (132). This constriction has been shown to be due to the presence of histamine as an impurity, and not to the pituitary extract itself (201, 234, 374). Glacial acetic acid, emetin hydrochloride, colloidal gold, lung extract, lutein, calcium lactate, caramel, copper sulphate and kaolin (164) belong to this class.

The drugs which cause broncho-dilatation may be classified under seven headings: those which 1, stimulate the sympathetic nerve endings; 2, inhibit the vagus; 3, poison or destroy the ganglion cells; 4, act directly on the muscle; 5, are reversible drugs, that is, those whose

activity is constriction unless a primary constriction be present, in which case they dilate; 6, constrict first, then relax; and 7, have an unknown site of action.

The drugs of class one, which stimulate the sympathetic endings, include adrenalin (17, 34, 146, 196, 203, 212, 279, 339, 342, 350, 351, 373, 450, 464), but claimed to be inert (94, 112); cocaine (17, 339, 450), but said also to be ineffective (197); caffeine (17, 339, 340, 341, 450), but the action is said to be central in origin (197), and relaxation is denied unless strong contraction is present already (279); calcium (260, 353, 354, 373); hordenine, hydrastinin, lodal (198, 200); epinine, choline chloride, trimethylamine hydrochloride, arterenol, homorenon (197); beta-tetra-hydronaphtylamine (200); ephedrine (4, 5, 69, 70, 269, 290, 291, 328, 442); primary and secondary amines (23).

Those of the second class, viz, those which paralyze the vagus endings, are: atropine (25, 45, 94, 109, 146, 189, 278, 279, 339, 350, 351, 356, 378, 449, 450); hyoscyamine, scopolamine, chloroform (94, 279); ether (45, 94, 279, 378); nicotine (45, 146, 450); lobelia (100); cocaine, aconitine (221).

Those of the third class, whose activity is upon the ganglion cells, are: lobelia (94, 279), but claimed to be inactive (450); gelseming (279); nicotine (94, 109, 279), although found to be inactive (450).

Drugs of the fourth class, those acting directly on the muscle, include: papaverine (272, 273, 278, 279, 340); tetrahydropapaveroline hydrochloride (241); chelidonin (159, 160, 161, 162, 279); pantopon (279); benzyl benzoate (275, 276, 279, 380, 474), but said to be inactive (283); benzyl alcohol (276, 279); benzyl nitrite (277, 279); benzyl succinate (277); benzyl acetate (274, 279); benzyl esters (274).

The fifth class have been termed reversible drugs by Golla and Symes, causing constriction unless the bronchi are already constricted, when they act as dilators. To this group belong epinine, ethylamine, methylamine, apocodeine, isoamylamine, tyramine (144), the last two mentioned showing this activity because of their stimulation of the adrenals, not because of direct action upon either muscle or nerve endings (197). Tyramine causes sometimes constriction, sometimes dilatation (436). Adrenalin falls in this class (144, 145), although this may be due to the fact that it is said to stimulate either the sympathetics or the parasympathetics depending upon which system exhibits the lower threshold of stimulation at the time of the exhibition of the drug (176, 345).

Class six comprises those drugs whose initial activity is constriction, followed by a relaxation. They are alcohol, chloral hydrate, urethane

(450); chloroform, ether, (279, 450); nicotine (197); curare, morphine (94). The action of nicotine is quite a complex one and not yet wholly explainable (197). Its relaxing effect is probably due to its stimulating action upon the adrenals (59, 197).

To class seven, the broncho-dilators whose point of action is unknown, belong a great many drugs. They are: urethane (94, 130, 279, 450); sodium iodide, amylnitrite, diuretin (339); sodium nitrite (339, 350, 351); alkalies in strong concentration, novocaine, stovaine, alypin, holocain, eucain, theophyllin, theobromine, xanthine, hypoxanthine, guanin, adenin, potassium ion (279); carbon dioxide, hydrogen ions, strychnine, emetin, morphine, ammonium ion (450); narcein, narcotin (272, 273, 279); cinnamein (278); nitrite ion, iodine (279), although their activity is denied (450); thyroid extract (144); hydrochinon (196); cocaine (17, 279, 339), but its effect is questioned (197); quinine (279, 450); hydrocyanic acid (94); veratrine (350, 351); copper sulphate in large quantities, colloidal iron (164); nitroglycerine, morphine, alcohol, nicotine (465).

It will be noted that in the above lists of drugs there are: 1, those whose activity is unquestioned; 2, those whose specific activity is denied; 3, those to whom the opposite activity is assigned by some authors, and 4, those which are claimed to be dilators by some, constrictors by others and ineffective by still other authors.

To the second class have been assigned physostigmine, ergotoxin, thebaine, peptone, histamine, heroine, codeine, whose activity as broncho-constrictors has been questioned on various grounds. Those drugs whose activity as broncho-dilators has been questioned are adrenalin, cocaine, caffeine, benzylbenzoate, epinine, and the nitrite ion.

To the third class belong bromine, quinine, pantopon, narcotine, curare, the acid ion, and the hydroxyl ion.

To the fourth class belong veratrin, morphin, and nicotine.

These apparent discrepancies may have their foundation in many things. The hydrogen ion concentration of the drug (279), the quantity used, the purity of the drug, the physiological condition of the musculature of the bronchi (such as profound relaxation or constriction), the effect of the drug used on other parts of the organism which in turn might influence the muscles (as the stimulation of the adrenals), the animal used, the use of intact or isolated muscles, the technique employed to determine the effect of the drug, and many other conditions not mentioned might influence the results, so that varying responses might easily be shown to the same drug.

Asphyxia. In connection with the dilating effect of adrenalin upon the bronchial musculature, the question of the effect of asphyxia upon the secretion of the adrenals is of interest. If asphyxia increased the output of adrenalin it would tend to act as a safety factor by thus insuring the dilatation of the bronchi and a consequent greater opportunity for ingress of air. There are two opposed schools of workers, some contending that asphyxia does increase the output of the adrenals, the others denying this. Many criteria have been used to establish the truth of the question, and it appears to be unsettled still. The usual method is to test the control animal, with adrenals intact, against the animal from which one adrenal has been removed, and the other denervated, since the secretion is stated to be liberated under the influence of the nervous system only (412). Asphyxia causes an increase in the adrenalin output when the denervated limb is used as a criterion (8, 9, 58, 182); also when the denervated heart is used as the test (9, 57, 60, 61, 135, 256, 398); when the rabbit's intestine is used (62, 85); when brain temperature is the standard (82); and when hyperglycemia is the criterion (52, 56, 106, 107, 215, 218, 334, 348, 475). Utilizing the "paradoxical pupil" as the test, the dilatation of which was claimed to be due to adrenalin (287), a number of workers claim that asphyxia increases the adrenalin output (91, 113, 166, 167, 168, 169, 170, 218, 219), while rise in blood pressure has been used to prove the same point (182, 192, 446, 447, 448). Flashman found that rats succumbed to anaphylactic doses of horse serum more rapidly if the adrenals were removed than did the controls, and this was supposed to be due to lack of adrenalin to alleviate the bronchial spasm, the anaphylactic dose having been shown to increase the adrenalin output (183).

From the histological side, evidence has been presented to show that after asphyxia there is a lower content of adrenalin in the suprarenal glands than is found normally (37, 213, 223, 407, 446).

On the other side of the question are ranged Gley and Quinquaud, and Stewart and Rogoff. Although the two former workers admit that the concentration of adrenalin is increased during asphyxia, they hold that this is of no significance in the blood pressure and cardio-vascular changes which follow (140, 141, 142, 143). Using the same criteria that have been used to prove that asphyxia increases the output of adrenalin, various workers have found no increase in adrenalin (151, 343, 409 to 425 inclusive).

It seems difficult to determine the reason for the diversity of results in these two schools of workers. May it be that some adrenalin is secreted

into the blood stream from the remaining chromaffin tissues, so that the animal supposed to be excluded from the effects of the adrenals is not in reality so? May the secretion, which had been supposed to be under the influence of the nervous system only, be elicited by lowered oxygen tension or increased carbon dioxide, or by some other chemical means? Ligation of the vein from the adrenal does not necessarily mean that the adrenal is cut off from the circulation, since there have been shown to exist anastomoses between the medulla and veins in the capsule (120) and between the adrenal and veins in the kidney (77). Hoskins, who has reviewed, in this journal, the literature on the subject, concludes that asphyxia does cause an increased secretion of adrenalin.

THE BRONCHIAL MUSCULATURE IN PATHOLOGICAL STATES. *Asthma.* With the fundamental pathology or the clinical manifestations of asthma this review is not concerned, but inasmuch as spasm of the bronchial musculature is one of its major features, mention must be made of it. A review of the literature reveals an attempt on the part of some to find the same underlying cause for all cases, whereas at least three distinct types emerge: those due 1, to reflex spasm caused by irritation, especially of the nasal mucosa; 2, to anaphylactoid reactions to certain proteins, and 3, to so-called essential hyperexcitability of the vagus. The result of these three conditions is a constriction or hypertrophy or both of the bronchial muscles. Some workers claim that the hypertrophy is the essential feature (187).

There is a great deal of evidence, experimental and clinical, to show that stimulation of the nasal mucosa may cause a reflex spasm of the bronchial muscles through the vagus nerve (1, 7, 38, 46, 102, 117, 123, 124, 128, 148, 190, 237, 270, 284, 375, 403, 433, 452, 454, 455).

Asthma due to sensitivity to foreign proteins has been recognized for some years, and a large literature has accumulated on this subject, to which only briefest reference can be made. These proteins apparently cause a typical broncho-stenosis or an edema of the mucosa, thus obliterating the bronchial lumen and accounting for most of the asthmatic symptoms (16, 29, 43, 55, 65, 73, 74, 93, 118, 131, 134, 147, 149, 210, 232, 260, 286, 353, 361, 377, 382, 437, 456, 457, 458, 459, 460, 461, 462).

In those cases of asthma in which nasal irritation and anaphylactoid phenomena can be excluded, and so fall into the third group, deficiency of calcium or excess of potassium ions seems to be the cause of the attacks. Calcium deficiency has been demonstrated (44, 331) and administration of calcium has afforded relief (261, 352, 353, 354). Pottenger (353) based his therapy on the following series of experimental work: Ringer

(370, 371, 372) had demonstrated the antagonism between calcium and potassium ions; Howell (184) and Howell and Duke (185) showed that stimulation of the vagus increased the potassium content of the heart but did not alter the calcium content and that sympathetic stimulation did not increase the potassium ions; other workers demonstrated the antagonism between these ions (92) and the increase of the calcium and potassium ions in the perfusate after stimulation of the sympathetic and vagus respectively (235, 477, 478). Pottenger concluded that in asthma due to a hyperexcitable vagus, administration of calcium would be beneficial by counterbalancing the excess of potassium thus liberated. Relief was afforded by this drug in those cases selected for its use.

Another mode of therapy, based on a consideration of the nerve supply of the bronchial muscles, has been the excision of the cervical sympathetic in the belief that most of the vagus fibers are found in the sympathetic chain (121, 209, 238); but there is lacking any real demonstration of the value of this procedure (137, 307, 220).

The change most characteristically found in the lungs of those who have died of asthma is an increase in the thickness of the walls of the bronchi and bronchioli of more than 0.2 mm. in outside diameter, as compared with similar structures in non-asthmatic persons. This hypertrophy involved all the components of the wall (187). It is of interest that they found little or no change in the smallest tubes of the bronchial tree, for Miller (319) states that "this arrangement" (in the form of a sphincter) "of the muscle about the atrial openings plays an important rôle in many cases of asthma by becoming strongly contracted during the asthmatic attack." It would appear from the autopsy findings that the site of the spasm was located much more centrally, in fact before the respiratory bronchiole was reached. It is not improbable that the point of constriction is the terminal bronchiole which has been stated to be the narrowest portion of the entire airway (188, 262). In anaphylaxis, the site of the spasm is located in the secondary and tertiary bronchi (393).

Anaphylaxis. In his review of the subject of anaphylaxis in the first volume of this journal, Wells (469) pointed out that its symptoms were different in differing species, and that these depended upon the distribution of the smooth muscle in the animals' organs. He says: "Stimulation of unstriped muscle is universal, and the symptoms observed depend upon the degree of development of these muscles, or their strategic location, in different organs of different species." Thus are accounted for the micturition, defecation, erection of hairs and exophthalmos seen

in anaphylaxis. In guinea pigs, because of a very rich supply of muscle in the bronchioles, the chief symptom is asphyxia, due to their constriction; in rabbits, in which the muscle coat of the pulmonary arteries is highly developed, distention of the right side of the heart and circulatory failure predominate, and in dogs, in which the hepatic veins are said to be unusually rich in smooth muscle, the effect of anaphylaxis is splanchnic and hepatic congestion, owing to the obstructed flow through the liver.

Only that aspect of the work which relates to the constriction found in the bronchial muscles can be referred to here. The phenomena of anaphylaxis in guinea pigs, having been shown to be connected with respiratory disturbances and associated with changes in the lungs (136) which were peripheral in origin (13, 14, 15, 31, 32, 33), (although there are workers who consider them to be central in origin—264) are due to the intense asphyxia produced by bronchiolar constriction (6, 393).

The next point of interest in this connection is whether the site of attack of the sensitizing protein is in the nerve ending or in the muscle cells. Much has been written on this and the evidence is not as yet sufficiently uniform to permit of final judgment. Observations on the effects of section of the vagi, or the administration of atropin in the prevention of the onset of anaphylactic symptoms have led some workers to conclude that the point of reaction is in the muscle cell itself (12, 88, 344, 392), while results obtained in other instances would indicate that the nerve ending was the susceptible structure (10, 11, 13, 165) inasmuch as atropine, supposed to exert its influence on the vagus endings, relieved or prevented the appearance of the bronchial spasms. Auer (10) suggests that the cause of this action of atropine is that the dose is sufficiently great to paralyse the nerve endings and then act directly on the muscle itself, producing dilatation. In this connection it must be remembered that Jackson (197) finds that atropin, after paralysing the vagus endings, acts on the muscle, causing further contraction.

Wells states that the "essential features of anaphylactic shock depend upon intracellular reactions between antibody and antigen that have been withdrawn from the circulation" and adds elsewhere that this reaction is peripheral, not central, in location. He does not state whether this intracellular point is the nerve ending or the substance of the muscle cell.

Other pathological changes. There is on record evidence that the quality or quantity, or both, of the bronchial muscles may be altered in pathological states. Hypertrophy of these muscles has been found in brown

induration of the lung (254, 369); in parasitic infection of the lungs (103, 429); in "desquamative pneumonia" (51); in asthma (187); while hypertrophy in some cases and atrophy in others has been described in emphysema (254). Pure atrophy in the walls of bronchiectatic cavities has been found (53, 254, 282, 363)—indeed atrophy is to be looked upon as the essential cause in the formation of the cavity (53, 54, 363). Some of the phenomena of pulmonary edema and functional dyspnea (191) and the accumulation of large masses of secretion in the bronchi in laryngeal diphtheria (53, 54) have been attributed to the impaired functioning of the bronchial muscles. Massive or atelectatic collapse of the lung has been ascribed to broncho-constriction following injury to the region of the vagus (383). The symptoms of anaphylactic shock have been discussed.

THE INTERSTITIAL MUSCULATURE OF THE LUNGS. It has been known for some time that there is, in the interstitial tissue of the lung, apart from the bronchial tree proper, a delicate system of muscle fibers. Baltisberger, in discussing this, has termed it the interstitial musculature, and has divided it into three parts: 1, the muscle fibers of the interlobular septa; 2, those of the bronchial and vascular sheaths, and 3, those of the visceral pleura. Collectively, it is an important element of the lung.

It is quite possible that some of the workers who found muscle in the walls of the alveoli were interpreting the strands which were lying in the septa or in the pleura as belonging to the air cells, which were adjacent to them. The interstitial muscle occurs in the form of a fenestrated membrane, or in the sheaths about the blood vessels, lymph vessels or bronchi, but yet distinct from the walls of these structures. Just what the function of these shreds of muscle may be is not at all clear. It is possible that they serve to diminish the lung volume. It would be only reasonable to assume that this muscle contracted synchronously with that of the airway with which it is connected by muscle strands. What rôle, if any, this interstitial muscle plays in abnormal conditions, or in disease, it is impossible now to state.

OUTLOOK. We have presented what we believe to be the modern conception of the airway, particularly of its terminals, and have fitted the bronchial musculature thereinto. We have made an attempt to analyze the respiratory form changes of these tubes, and have tried to visualize the participation of the muscles in the processes of filling and emptying the terminal air-chambers. We have sought to throw light on certain of the more unusual, or emergency, uses of these muscles, and have noted some of the pathological changes which occur in them.

Much remains to be said, and if this brief review serves merely to concentrate attention upon this field, and to stimulate others to undertake the cultivation of it, the work will have been justified. Certain it is that the problems concerned with this musculature are inviting ones: and that they involve generalizations of real significance in clinical medicine and public health is evident from the closeness of their bearing upon such vital studies as those of pneumonia and tuberculosis. The future looks very bright. Recent years have seen the introduction of methods which, although only in their infancy, yet have added much to our store of knowledge. Among these perhaps the most valuable is roentgenoscopy; and the refinements of technique now coming in, such as stereoscopic films and the injection of radio-opaque materials, like lipiodol, into the bronchial tree, have only begun to yield their harvest of new facts. In microscopy, too, we have the stereo-binocular instrument, whose use has already shed so much light upon the structure of the lung, and we have a steadily increasing wealth of technical maneuvers which bids fair to open up for us truly a new world in this organ. And more, perhaps, than all of these is the strong reawakening of interest in this field, and the small army of keen workers who are laboring in it. On this continent we are indebted more to the work of William Snow Miller than to that of any other man for our increasing knowledge of the structure of the lung, and it is a real pleasure to pay tribute to his worth as a discoverer of new facts in lung anatomy. His work illustrates the importance of clear objectivizations in the solution of physiological problems in this field; of the essential value of the anatomical mode of thought.

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APPENDIX. Since writing the above the paper by Heinbecker (492) has appeared. By x-ray pictures of lipiodolized bronchi and bronchioli in the human lung he demonstrates an increase in width at the end of full inspiration as contrasted with the end of full expiration, thus confirming my own conclusions for the larger tubes. During quiet breathing this movement was slight, or undemonstrable. Inspiratory increase in bronchial length was reported. He thinks that some of the longer bronchi, particularly those to the lower lobes, may be actually narrowed during the early part of inspiration, but his evidence for this is unconvincing.

THE RESPIRATORY QUOTIENT

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1. PRINCIPLES. The respiratory quotient is the most direct measure, so far devised, of the relative quantity of foodstuffs entering into the metabolism of the human or animal organism. To this it owes its chief importance. A far simpler method, of course, is to measure the three main constituents of the diet, assuming that the quantities utilized in a given period are the same as those ingested. This assumption, however, neglects the fact that the body is a commodious storehouse of foodstuffs, which may be laid down or withdrawn according to current needs. The degree to which the diet may differ from the utilization has been shown elsewhere. (Richardson and Mason, 1923; Richardson, 1923.) Only when a measured diet is given over long periods, during which there is no change of weight, can accurate conclusions be drawn. Even then there is a possibility of one foodstuff replacing another in the body.

Higher organisms, although able to do work anaerobically for a short time, derive their energy in the long run solely from oxidation. If the quantities of oxygen and carbon dioxide involved can be measured, an

estimate may be made, not only of the total quantity of heat evolved, but also of the quantities of protein, fat and carbohydrate consumed. This is accomplished by means of the respiratory quotient, with the aid of the urinary nitrogen. The former may be defined as the relation, expressed in volumes, between the oxygen consumed in the reaction and the carbon-dioxide given off as a result. Its significance is based fundamentally on Avogadro's law, by which equal volumes of all gases contain the same number of molecules, or, in other words, equal numbers of molecules of all gases occupy the same volume. When a carbohydrate such as glucose, $C_6H_{12}O_6$, is oxidized, all of the oxygen utilized goes to form carbon-dioxide, and for every mol. of oxygen one mol. of carbon-dioxide results. This is evident from the equation:

$$C_6H_{12}O_6 + 6O_2 = 6H_2O + 6CO_2$$

$$\text{R.Q.} = \frac{\text{6 mol. } CO_2}{\text{6 mol. } O_2} = \frac{\text{6 vol. } CO_2}{\text{6 vol. } O_2} = 1.0$$

When fat is utilized some of the atmospheric oxygen goes to form water, and the respiratory quotient is less than 1.0. The reaction for tripalmitin may be given as an example: (Du Bois, 1927)

$$C_{51}H_{98}O_6 + 72.5O_2 = 51CO_2 + 49H_2O$$

$$\frac{\text{51 mol. } CO_2}{\text{72.5 mol. } O_2} = \frac{\text{51 vol. } CO_2}{\text{72.5 vol. } O_2} = 0.703 = \text{R.Q.}$$

For mixtures of fats the quotient is usually considered to be 0.707. A table of respiratory quotients of other food materials will be found on page 78.

Except in rare instances both carbohydrate and fat enter into the metabolism, hence the quotient is neither unity nor 0.707, but somewhere between. Assuming for the sake of simplicity the oxidation of a mixture consisting only of these two substances, the exact proportion of each may be determined, using the table first worked out by Zuntz and Schumburg (1901). Lusk's (1924) more recent modification is given in part below.

For example, given the oxidation of 100 calories of a mixture of carbohydrate and fat with a respiratory quotient of 0.90. We find from the table that 67.5 per cent or 67.5 calories are derived from carbohydrate, and the remaining 32.5 from fat. Since each gram of higher carbohydrate yields, on the average, 4.1 calories, and each gram of fat 9.3

calories, we conclude that 16.45 grams of the former and 3.5 grams of the latter have been consumed.

As implied above it is necessary to know the total calories which are given off by the oxidation. This may be calculated from the volume of oxygen consumed, for every litre of which a certain number of calories are given off, depending on the level of the respiratory quotient. The exact number may be read off from the table. If 20.3 litres of oxygen were consumed with a respiratory quotient of 0.90, the calories would be 20.3×4.924 or 100.0.

The above discussion neglects the fact that protein always takes part in the metabolism, the exact extent being determined by the total nitrogen of the urine. From the elementary composition of this food, it has been calculated (see Lusk, 1919, p. 60 and 62) that for every gram of urinary nitrogen 8.45 grams of oxygen are consumed, 9.35 grams of carbon-dioxide liberated, and 26.5 (large) calories given off. Lusk (personal communication) has recently corrected the value for oxygen, which should therefore read 8.49. Using the above figures the share of protein in the oxidation may be determined, and subtracted from the total, leaving the quantities due to carbohydrate and fat. The calculation of the data obtained with the respiration calorimeter in the case of George H., April 23, 1923 (Richardson and Ladd, 1924) may serve as an example.

Urinary nitrogen		0.202 gm. per hr.	
Total CO_2 excretion		16.29 gm. per hr.	
Total O_2 consumption		14.29 gm. per hr.	
Protein calories		$0.202 \times 26.51 = 5.35$ cal.	
Protein CO_2		$0.202 \times 9.35 = 1.89$ gm.	
Protein O_2		$0.202 \times 8.49 = 1.71$ gm.	
Total CO_2	16.29	Total O_2	16.00
Protein CO_2	1.89	Protein O_2	1.71
Non-protein	14.40		14.29
Change to litres	× 0.5089		× 0.6997
	7.33		10.0
Non-protein R.Q.	0.733		

Interpolating in table 1 it may be seen that for a non protein respiratory quotient of 0.733 each litre of oxygen is the equivalent of 4.718 cal.

$$10.01 \times 4.718 \text{ cal.} = 47.2 \text{ non-protein cal.}$$

TABLE 1

Analysis of the oxidation of mixtures of carbohydrate and fat

R.Q.	PERCENTAGE OF TOTAL HEAT PRODUCED BY:		CALORIES PER LITER O_2	
	Carbohydrate (1)	Fat (2)	Number (3)	Logarithm (4)
0.707	0	100.0	4.686	0.67080
0.71	1.10	98.9	4.690	0.67114
0.72	4.76	95.2	4.702	0.67228
0.73	8.40	91.6	4.714	0.67342
0.74	12.0	88.0	4.727	0.67456
0.75	15.6	84.4	4.739	0.67569
0.76	19.2	80.8	4.751	0.67682
0.77	22.8	77.2	4.764	0.67794
0.78	26.3	73.7	4.776	0.67906
0.79	29.9	70.1	4.788	0.68018
0.80	33.4	66.6	4.801	0.68129
0.81	36.9	63.1	4.813	0.68241
0.82	40.3	59.7	4.825	0.68352
0.83	43.8	56.2	4.838	0.68463
0.84	47.2	52.8	4.850	0.68573
0.85	50.7	49.3	4.862	0.68683
0.86	54.1	45.9	4.875	0.68793
0.87	57.5	42.5	4.887	0.68903
0.88	60.8	39.2	4.899	0.69012
0.89	64.2	35.8	4.911	0.69121
0.90	67.5	32.5	4.924	0.69230
0.91	70.8	29.2	4.936	0.69339
0.92	74.1	25.9	4.948	0.69447
0.93	77.4	22.6	4.961	0.69555
0.94	80.7	19.3	4.973	0.69663
0.95	84.0	16.0	4.985	0.69770
0.96	87.2	12.8	4.998	0.69877
0.97	90.4	9.58	5.010	0.69984
0.98	93.6	6.37	5.022	0.70091
0.99	96.8	3.18	5.035	0.70197
1.00	100.0	0	5.047	0.70303

Formula for Column (R.Q. = R)

(1) $$\% = \frac{504.7\ (R - 0.707)}{5.047\ (R - 0.707) + 4.686\ (1.00 - R)}$$

(2) $$\% = \frac{468.6\ (1.00 - R)}{5.047\ (R - 0.707) + 4.686\ (1.00 - R)}$$

(3) $$\text{Calories} = 4.686 + \frac{R - 0.707}{0.293} \times 0.361$$

(4) Logarithm = log of Column 3

Abbreviated from Lusk (1924)

With a R.Q. of 0.733, 90.5 per cent of these calories are derived from fat and 9.5 per cent from carbohydrate.

Fat, cal. = 47.2 × 90.5 per cent = 42.7 cal.
Carbohydrate, cal. = 47.2 × 9.5 per cent = 4.5 cal.
Fat, gram. = 42.7 ÷ 9.3 = 4.59 grams
Carbohydrate, gram = 4.5 ÷ 4.1 = 1.09 gram
Protein, gram = 5.35 ÷ 4.1 = 1.30 gram

Du Bois (1924) has devised a graph which may be used in place of the Zuntz-Schumburg table. Workers who are accustomed to the latter should bear in mind that in the graph the percentages are expressed in terms of total, rather than non-protein, calories. The data required are the total respiratory quotient, the nitrogen calories, and the total calories. This method has the further advantage that it permits graphic representation of the data, not only in a single experiment, but also in a series. This has been well illustrated in a paper by Du Bois (1924–25). The late A. M. Michaelis (1924) published an extension of the principle, by which the entire calculation can be performed graphically, knowing only the nitrogen of the urine, the total oxygen consumption, and the gross respiratory quotient.

Whatever the method of calculation, it should be done whenever the data warrant it. The results are not only more accurate, but also more illuminating. Considerable information may be obtained, however, without the above corrections. This is because relatively little protein is oxidized, and that little has a respiratory quotient of 0.8016 (Lusk, 1919) which is near the usual figure for the body as a whole.

Given, then, an organism oxidizing a mixture of protein, fat and carbohydrate, one determines the oxygen consumed, the carbon-dioxide eliminated, and the nitrogen of the urine. Applying to these figures the calculations outlined above, one obtains the quantity of protein, fat and carbohydrate which were actually concerned in the oxidation during the period of observation.

2. Apparatus. Many types of apparatus in use for the determination of the total metabolism can be used for the respiratory quotient as well.

One of two principles may be utilized, the "closed circuit" or the "open circuit." In the former the same air is used over and over again, and the entire bulk of the gases concerned in the respiratory exchange is measured. With the "open circuit" the subject receives with each breath a renewed supply of atmospheric air, and for the most part only

a minute fraction of the outgoing air is analyzed. With either method the subject may be outside or within the apparatus, that is to say, he may be connected by means of a mask or mouthpiece, or enclosed within an air-tight chamber.

The principle of the closed circuit may be most readily understood from a description of the Benedict Universal Respiration Apparatus, (Carpenter, 1915), although the latter is no longer in extensive use in the form described below. This apparatus is a simplification of the respiration calorimeter, the history of which may be found elsewhere. (Lusk, 1915 *a*, 1919, 1922.) The subject breathes through a mouth-piece from a current of air which is propelled by means of a motor and blower. The air then passes through concentrated sulphuric acid to remove the moisture, and afterwards through the carbon-dioxide absorber. This consists of a special bottle full of soda-lime, and another of concentrated sulphuric acid, the latter to retain any water that may be given off from the moist soda-lime. The two are weighed together, the increase in weight being due to carbon-dioxide. To provide the elasticity necessary for respiratory movements a small spirometer is is introduced into the system, or else a large aperture covered with a bathing-cap. Oxygen is admitted as needed, either from a weighed cylinder, or through a Bohr meter, either method serving to determine the quantity admitted into the apparatus. In order that this quantity should be equal to the oxygen consumed, the volume of the entire system, including the lungs of the subject must be the same at the end of the determination as at the beginning. The spirometer bell or bathing cap must therefore be at the same level, and respiration at the same phase. To aid in fulfilling the latter requirement, a T-valve is provided by which connection with the apparatus can be made or broken instantaneously.

The principle of the respiration calorimeter is the same as the foregoing, insofar as the gaseous exchange is concerned, except that the subject, instead of using a mouth-piece, is enclosed in an air-tight chamber. The volume of the latter is so considerable, that corrections must be made for changes in temperature, pressure and composition of the residual air. This apparatus has been exhaustively described by Lusk (1915a) and by Riche and Soderstrom (1915). The cot-chamber apparatus of Benedict and Tompkins (1916) is essentially the same, except that no provision is made for the direct measurement of heat, which, however, does not concern us here. The same method has been adapted to the study of infants by Benedict and Talbot (1914) (1915).

All apparatus of the open-circuit type is similar in that the volume of outgoing air is measured, and one or more samples subjected to gas analysis. The carbon-dioxide eliminated is determined by the difference between the quantity expired and the quantity inhaled; the oxygen consumption in a similar manner. Whenever the respiratory quotient is less than 1.0 the air (corrected for vapor pressure) loses volume through respiration, and this shrinkage must be included in the calculations. Thus:

$$\frac{\%CO_2 \text{ out} \times \text{Vol. out} - \%\ CO_2 \text{ in} \times \text{Vol. in}}{\%O_2 \text{ in} \times \text{Vol. in} - \%\ O_2 \text{ out} \times \text{Vol. out}} = \text{R.Q.}$$

where $\%\ CO_2$ in = percentage of CO_2 of ingoing air
$\%CO_2$ out = percentage of CO_2 of outgoing air
Vol. in = volume of the ingoing air
Vol. out = volume of the outgoing air
$\%\ O_2$ in = percentage of O_2 in ingoing air
$\%\ O_2$ out = percentage of O_2 in outgoing air

The volume of the expired air is measured and reduced to volumes dry at 0°C. and 760 mm. Correction for change in dry volume is made by means of the alteration in percentage of nitrogen, as recently described by Dautrebande (1926) and Du Bois, (1927, p. 93).

Types of apparatus employing this principle vary in the manner of propelling the air-current, measuring the volume of the outgoing air, and connecting the subject to the apparatus. In the method in common use in this country the lungs provide the motor power, and the current is given the proper direction by means of valves. The subject uses a mask or mouth-piece. The expired air is caught in a large, delicately balanced spirometer, such as that designed by Tissot (1904), so calibrated that the volume can be read off. Any standard form of gas-analyzer may be employed, such as the Haldane (1912) or the modification introduced by Henderson (1918). For a detailed description of this method with practical improvements, the article of Bailey (1921) may be consulted. If portability is desired, the air may be collected in one or more Douglas bags (Douglas, 1911) and afterwards metered. In the older Zuntz-Geppert apparatus (Magnus-Levy, 1894), (Carpenter, 1915), valves and mouthpiece are likewise employed, but the outgoing air is passed through a meter, provision being made for taking continuous samples. In the Jaquet type (Jaquet, 1903), (Hassebalch, 1904), the subject is enclosed in a chamber through which air is driven. Here the large volume of the air-current requires the use of a meter and sampling-device. As in other forms of chamber apparatus, correction

is necessary for change in true volume and composition of the air in the system.

Benedict (1925b) has recently devised an apparatus of great promise, in which the gravimetric determination of carbon-dioxide is combined with gas-analysis.

For small animals the standard method is that of Haldane (1892). This is an open circuit method in which all the determinations are made by weight. The animal is enclosed in a small chamber and receives a current of dry air, free of CO_2. The CO_2 and water of the outgoing air are absorbed in weighed tubes. Oxygen is determined indirectly by the change in weight of chamber and animal combined.

Most if not all of the above devices can be used for the study of exercise. In the respiration calorimeter or cot-chamber apparatus, the degree of the exertion is somewhat restricted because it is necessary for technical reasons to keep the humidity within limits. Krogh and Linhard (1920) have used the open circuit with a chamber and obtained accurate results. In this country it is customary to use two spirometers alternately, or one very large one. Hill, Long and Lupton (1924b) employed a number of Douglas bags, one after another. Furusawa (1926) describes a calculation by which the respiratory quotient can be determined in short successive periods using a single spirometer.

Both principles of determining the respiratory quotient have their advantages and defects. In the closed circuit method the apparatus must be completely air-tight, because any leak produces an error of equal magnitude in the oxygen consumption. The Benedict Universal apparatus (Carpenter, 1915) is open to this objection, and as pointed out by the inventor, has the further disadvantage that the exact volume of the system at any given moment cannot be determined because it includes the lungs of the subject. It has therefore been superseded by the Benedict-Collins and Benedict-Roth machines which do not, however, permit the measurement of the respiratory quotient. With the respiration calorimeter and cot-chamber apparatus the total volume of the system undergoes no change. Furthermore, the absence of leaks can be demonstrated by means of alcohol checks. A technical drawback has been described by Lusk (1915a). Any movement of the patient at the end of an experimental period causes a negative error in the oxygen consumption for that period, and a positive error in the next. If, however, the movement occurs at the beginning of the observation a fresh start may be made, and if at the end, a new period may be observed. The error therefore produces only changes from hour to hour, but does not affect the average for the whole observation.

With the open-circuit method leaks are less serious. The accuracy of the method depends upon the gas-analysis. Small samples only are taken, as contrasted to the closed method, where the entire volume of oxygen or carbon-dioxide is measured. The gas-analyzer must be checked at frequent intervals by analysis of atmospheric air. When a chamber is used, the outgoing air is of so large a volume, that it differs little from the atmosphere. Highly sensitive gas-analyzers are therefore required, such as the Sonden-Pettersson (Sonden, 1889) (Benedict, 1912) or that of Carpenter (1923) or Krogh (1920).

The chief advantage of the use of mask or mouth-piece is simplicity and compactness. These appliances have the disadvantage that they do not always fit tightly. The leakage is not easy to detect, although the method suggested by Benedict is of value with apparatus employing a small spirometer and a graphic method of registration. If a small weight be placed on the bell of the spirometer, the leak is revealed by a change in the incline of the curve. The discomfort of the mouthpiece makes for short observations. The most serious drawback, however, is its tendency to stimulate the respiration, and cause a large positive error in the respiratory quotient. A chamber does not have this tendency. Such over-ventilation as occurs is necessarily transient, and must give way to the reverse process within the period necessary for this type of apparatus.

Any apparatus employing a chamber can be readily tested for leaks by burning in it a known quantity of alcohol, and comparing the actual results with the theoretical. This has been done as a routine with the respiration calorimeter and cot-chamber types. Carpenter and Fox (1923) have invented a device by which the same principle may be applied to any type of apparatus. This deserves wide application, but, as suggested above, cannot demonstrate the tightness of the connection between subject and apparatus.

Measurement of breathing is not confined to intact animals, but may be applied to organs as a whole or to thin slices of tissues. The organ may be perfused, or studied in situ without interruption of the blood supply, the latter being the more desirable. The respiratory quotient is determined by analysis of the nutrient fluid before and after its passage through the organ, or by means of simultaneous samples of arterial and venous blood. Under ideal conditions (which seldom exist) the respiratory quotient is simply the increase in the percentage of carbon-dioxide divided by the decrease in the percentage of oxygen. Use of this formula involves assumptions which become evident when it is expanded.

The principles involved are exactly the same as those used in the measurement of the gaseous exchange of the intact animal by air analysis. The carbon dioxide evolved is equal to the output minus the amount supplied in the nutrient fluid, or per cent (by vol.) of CO_2 $\times$ vol. of outflow minus per cent CO_2 of the inflow $\times$ the vol. of the inflow. This quantity is the CO_2 of respiration, plus the amount expelled by acid formation (see section 3). The following formula includes this correction.

$$\text{R.Q.} = \frac{\%\ CO_2 \text{ out} \times \text{Vol. out} - \%\ CO_2 \text{ in} \times \text{Vol. in} - CO_2 \text{ expelled}}{\%\ O_2 \text{ in} \times \text{Vol. in} - \%\ O_2 \text{ out} \times \text{Vol. out}}$$

where the symbols have the same meaning as in the formula above (p. 67).

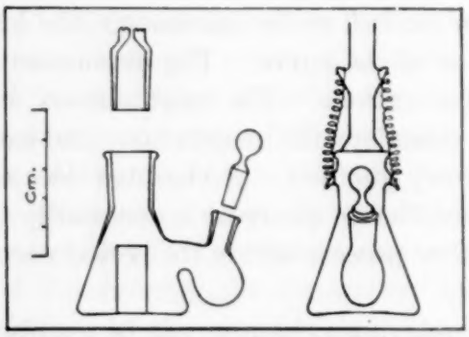


Fig. 1. Vessel of Barcroft-Warburg apparatus for manometric determination of micro-respiration.

If carbon-dioxide is retained instead of expelled, the sign is changed. If Vol. in = Vol. out, and CO_2 expelled = 0,

$$\text{R.Q.} = \frac{\%\ CO_2 \text{ out} - \%\ CO_2 \text{ in}}{\%\ O_2 \text{ in} - \%\ O_2 \text{ out}}$$

which is the simplified formula first mentioned. In perfusion experiments the expanded formula might be used since the inflow and outflow can be measured. With the circulation of the organ intact, this is not so easy, but the correction may be made by noting the change in concentration of hemoglobin or solids in the blood. In both methods the change in carbon-dioxide content of the preparation must be estimated by determining the carbon-dioxide dissociation curve of the ingoing and outgoing fluid.

These methods have the advantage that they confine the observations

to a single organ. The changes in gaseous content are so small as to tax the accuracy of the apparatus, and may be rivalled in magnitude by the necessary corrections. The technique therefore requires unusual skill, and even then the margin of error is wide.

The respiratory quotient of bits of living tissue can also be determined, for instance, by means of the manometric apparatus of Barcroft and Kato (1915) as modified by Warburg (1923). Since this method involves a minimum of trauma to the tissue, and is being extensively used, it is here described. Figure 1 is a diagram of a single unit, several of which are required. Tissue from a freshly killed animal is laid on filter paper and cut thin by means of a razor blade. The thickness of the section is calculated from its area and weight, and, as shown by Warburg's formula (1923), must not exceed a certain limiting thickness, depending on the oxygen tension, the rate of oxygen consumption, and the diffusion constant of the gas for tissue. Sections are put in mammalian Ringer solution (see below) and transferred to the main portion of the vessel shown in figure 1. This vessel contains Ringer solution (previously boiled to drive off CO_2) to which a phosphate mixture (also boiled) is added to make a concentration of molar 1:150 and a pH of 7.4. It is equilibrated against an atmosphere of pure oxygen and transferred to a water bath at 37.5°C., the manometer remaining outside. Apparatus is provided for causing the manometer to move like an inverted pendulum, thereby promoting free diffusion of gases. The manometer contains Brodie's solution, (Warburg 1923) which gives an excursion of 10,000 mm. per atmosphere. The volume of the system is kept constant by means of a screw which exerts pressure on the rubber reservoir.

Pressure change is converted into volume change by means of a simple proportionality factor, which differs according to the gas measured and the volume of air in the vessel. In calculating this factor the formula of Warburg (1923) is used, which is derived from the gas laws.

For the determination of a quotient 4 or 5 vessels are required. No. 1, a blank, serves to equalize changes of barometer or room temperature. Its readings are subtracted algebraically from those of the experimental vessels. No. 2 is for oxygen consumption. A normal solution of KOH is put in the central well to absorb carbon-dioxide, and the pressure change is therefore assumed to be due to oxygen alone. No. 3 is for the determination of carbon-dioxide. The KOH is omitted, and the pressure change is the resultant between absorption of oxygen and evolution of carbon-dioxide. The former is calculated from the data obtained from

vessel 2, including the dry weight of the tissue, and subtracted algebraically from the observed change, giving the variation due to carbon-dioxide. This multiplied by the vessel-constant, equals the volume of carbon-dioxide. Before the final reading is taken the dilute HCl contained in the sac is inverted into the main vessel, thereby expelling the carbon-dioxide. The figure obtained therefore includes not only the expired gas, but that which was preformed as well. In order to allow for the latter, two additional determinations are required. Vessel 4 contains tissue and Ringer-phosphate, vessel 5 the fluid alone. No KOH is used, but the sac contains HCl. These are inverted early in the experiment, and the volume of carbon-dioxide is measured, giving the quantity preformed in the solution, and by subtraction the amount in the tissue, referred to milligrams dry weight. The appropriate amounts are deducted from the carbon-dioxide as measured in vessel 3, leaving the quantity due to respiration. Details of the calculation are appended as they have not been assembled elsewhere.

Manometric method. May 23, 1927. Kidney of normal dog. The Ringer solution was made up as follows:

NaCl	9 gm. per litre	1000 cc.
KCl	11.5 gm. per litre	20 cc.
CaCl	12.2 gm. per litre	20 cc.

The depression of the freezing point of this solution is 0.56°.

To 9 cc. was added 1 cc. of m/15 sod. phosphate mixture, pH 7.4 (2 cc. of acid phosphate to 8 of basic). Glucose was added dry to make 0.2 per cent; this is optional.

Determination of vessel constant; vessel 3.

	cu.mm.
Total volume of vessel and capillary up to 150 mm. mark, as determined by calibration with mercury	25,500
Volume of Ringer solution in vessel	3,000
Volume of acid (2.5 N HCl in sac)	200
Total volume of fluid	3,200
Volume of vessel and capillary minus volume of fluid	22,300

$$K = \frac{V_g \frac{273}{T} + V_f \alpha}{10{,}000} \text{ (Warburg, 1923)}$$

where K = constant
Vg = volume of gas in vessel to 150 mm. mark.
T = *absolute* temperature of bath
V_f = total vol. of fluid
α = Bunsen coefficient of solubility at 37.5°C. ($\alpha_{O_2} = 0.024$) ($\alpha_{CO_2} = 0.561$)

$$K_{O_2} = \frac{22{,}300 \times \frac{273}{310.5} + 3{,}200 \times 0.024}{10{,}000} = 1.97$$

$$K_{CO_2} = \frac{22{,}300 \times \frac{273}{310.5} + 3{,}200 \times 0.561}{10{,}000} = 2.14$$

(Change in volume of fluid changes the constant.)

Volume of oxygen = pressure change due to oxygen (h_{O_2}) × K_{O_2} and Vol. $CO_2 = h_{CO_2} \times K_{CO_2}$

	VESSEL				
	1 Thermo-barometer	2 O_2	3 O_2 and CO_2	4a Preformed CO_2	4b (later)
Reagent		0.5 cc. N/1 KOH in well	0.2 cc. 2.5 N HCl in sac	0.2 cc. 2.5 N HCl in sac	0.2 cc. 2.5 N HCl in sac
Ringer phosphate-glucose	3 cc.	3 cc.	3 cc.	3 cc.	3 cc.
Tissue, mgm. dry	0	11.67	9.82	8.76	0
K_O		1.79	1.97		
K_{CO_2}			2.14	2.11	2.11
h(change of pressure)—mm.		−173.5	−10.0	+14.2	+6.0
Time of measurement		2 hr.	2 hr.	start	at will
Remarks			Acid from sac poured in at 2 hrs.	Acid poured in at start	Acid poured in

*Calculation**

Vessel (2)

$$\frac{\text{Fall of pressure} \times K_{O_2}}{\text{wt. of tissue}} = \frac{173.5 \times 1.79}{11.67} = 26.6 \text{ cu. mm.} = Q_{O_2} \times 2 = O_2 \text{ per dry mgm. per hr.} \times 2$$

Vessel 4a

Rise of pressure × K_{CO_2} = 14.2 × 2.11 = 29.9 = preformed CO_2 in tissue and fluid

Vessel 4b

Rise of pressure × K_{CO_2} = 6.0 × 2.11 = $\frac{12.7}{17.2}$ cu. mm. = preformed CO_2 in fluid alone

*By slide-rule.

Divided by wt. of tissue, 8.76 — 1.97 cu. mm. preformed CO_2 per mgm. tissue

Vessel 3

$$\frac{(Q_{O_2} \times 2) \times \text{wt. of tissue}}{K_{O_2}} = \frac{26.6 \times 9.82}{1.97} = -132.8$$ = calculated fall of pressure due to O_2

-10.0 = actual fall

122.8 = Rise of pressure due to CO_2

$\times K_{CO_2} = 122.8 \times 2.14 =$ 263.0 cu. mm.

Preformed CO_2 per mgm. $\times$ wt., $1.97 \times 9.82 =$ 19.3

243.7

Preformed CO_2 per 3 cc. Ringer sol. = 12.7

231.0 cu. mm.

Divide by wt. of tissue, $\frac{231.00}{9.82} =$ 23.55 = $Q_{CO_2} \times 2$ or CO_2 in cu. mm. per dry mgm. per hr. $\times 2$

$\div 2$

$\frac{26.6}{2}$

11.78 = Q_{CO_2}

13.3 = Q_{O_2}

0.855 = R.Q.

The accuracy of the method for the kidney of the white rat was demonstrated by Richardson, Loebel and Shorr (1926), who found an average error in the quotient of ± 0.014. On the whole these results compare favorably with those obtained with the intact animal, although an occasional experiment may diverge for no obvious reason. Extreme care in the preparation of the vessels is required, as even a small trace of acid is fatal to accuracy.

Excised tissue has the advantage that the system is simpler and more readily controlled than with the intact animal. Correction for changes in the carbon-dioxide content of the preparation is inherent in the method, thereby eliminating a prolific source of error.

3. Critique. Even when the apparatus is technically correct, errors of interpretation may arise. The quotient as measured may not reflect concurrent oxidation. In this event, it is convenient to designate the observed quotient as the measured quotient, and the supposed quotient due to oxidation as the metabolic quotient. Similarly Takane (1926) has distinguished between apparent and true quotients. The latter is not always the same as the measured value, as for instance when carbon-dioxide is retained or released during the observation. The net quantity of oxygen in loose combination is not thought to vary

in short periods, but changes in the carbon-dioxide content may cause enormous modifications in the quotient. Mere increase in the ventilation of the lungs, such as frequently occurs with untrained subjects, is sufficient to blow off great quantities of this gas (Dautrebande, 1926). The use of a mask or mouthpiece sometimes raises the respiratory quotient to an absurd figure. Fortunately this process is short-lived, and in any long observation must equalize itself.

Another factor which causes a progressive change in the total quantity of carbon-dioxide in the body is the production or removal of lactic acid. This is evolved in muscular contraction, and removed during recovery. These two processes occur almost simultaneously, but when the demand for energy exceeds the quantity which can be supplied by oxidation, lactic acid accumulates in the body. As a result the respiratory centre is stimulated, ventilation of the lungs increases, and carbon-dioxide diffuses off. When the exercise ceases, the acid is removed, chiefly by synthesis into glycogen. Energy for this conversion is obtained by the oxidation of about $\frac{1}{5}$ of the lactic acid, or the equivalent in other foodstuffs (Hill, Long and Lupton, 1924a). Whether oxidized or synthesized the acid leaves free base behind, which immediately combines with carbon-dioxide, causing a retention of the latter and a depression of the respiratory quotient. To avoid this effect of acid one of two methods may be employed. Either the exercise may be prolonged until a steady state is reached, in which the production of acid is equalled by its removal, or else the observation may cover the entire period, from the beginning of exercise to complete recovery. It is desirable that the alkali reserve of the blood, as shown by the carbon-dioxide combining power of the plasma, should be determined, and found to be the same at the end of the observation as at the beginning.

The considerations mentioned in connection with lactic acid apply when any other fixed acid is thrown into the metabolism, as in diabetes. In this disease keto-acids are often produced, and cause an increase in the quotient when the formation exceeds the removal, or a depression when the reverse is the case.

When formation and removal are equal, the quotient is not affected by the acid, provided that it is taken away as free acid and not as salt. Suppose, for instance, that a man undergoes a respiratory observation of 3 hours during which he forms 1 gram of beta-hydroxy-butyric acid, and eliminates 1 gram of this acid in the urine. There would be no net change in the quantity of acid in the body, and no effect on the respiratory quotient. If, however, the acid were excreted in combination with

base, the removal of the latter would cause a relative acidosis, and a rise in the measured respiratory quotient. A constant rate in the production of ketones is therefore no guarantee that the measured respiratory quotient is the same as the metabolic. It is best to rely on the alkali reserve of the blood, which should be the same at the end of the observation as at the start.

In severe nephritis there is a retention of fixed acid in the blood, accompanied by hyperpnea, which must cause an undue increase in the respiratory quotient.

It is to be especially noted, with respect to all of the processes mentioned above, that the measured quotient is displaced from the metabolic value, only in case of a net change in the carbon-dioxide content of the body. A second point to be emphasized is that the quotient for the complete process is the same, regardless of the intermediary process. For example, the supposed conversion of fat into carbohydrate could have no effect on the respiratory quotient, unless some of the material thus formed were laid down in the body (or excreted) during the experiment.

Respiratory quotients obtained from isolated organs or tissues should be doubly scrutinized for reasons to be detailed in section 9.

The respiratory quotient is modified in accordance with a different principle by the conversion of carbohydrate into fat, and the deposit of the latter in the tissues. The evidence for this has been detailed by Lusk (1919) and Du Bois (1927). Briefly, more carbon is laid down than the glycogen deposits can accommodate, and the respiratory quotient rises well above unity, values of 1.5 having been observed. Presumably the same occurs in man, but chemical determinations are naturally lacking, and the quotient has not been observed to rise as high. After the ingestion of 100 or 200 grams of monosaccharide it may be of the order of 1.10.

Theoretical equations for this process have been worked out by Meissl (1886) and Bleibtreu (1901). The simplest one, that given by Lusk (1919) for the conversion of glucose into fatty acid, follows:

$$4\ C_6H_{12}O_6 + O_2 = C_{16}H_{32}O_2 + 8\ CO_2 + 8\ H_2O$$

This assumes the conversion of the whole bulk of the glucose into fatty-acid, without wastage, other than carbon-dioxide and water. The respiratory quotient of the reaction is 8.0, hence it would require only a little conversion to produce a marked effect on the quotient.

If the reverse process, the conversion of fat into carbohydrate, occurs,

it would be expected to have the opposite effect, namely, a fall in the quotient.

Another complicating factor, the incomplete oxidation of foodstuffs, will be considered in section 8.

4. THE CHARACTER OF THE CATABOLISM IN NORMAL RESTING INDIVIDUALS WITHOUT FOOD. Since the character of the catabolism is dependent upon the food taken during the hours preceding the observation it is necessary to select some standard time, as is done with the basal metabolism. It is convenient, as with the latter, to begin about fourteen hours after the last meal. The subject is therefore studied at rest in the morning, having taken no food since supper of the preceding day. When the observation conforms to the above conditions it is known as basal, and this term may be extended to include the respiratory quotient as well as the caloric output.

Even the basal quotient is affected by the preceding diet. After ordinary mixed fare (Du Bois, 1927, p. 97), however, it usually lies between 0.80 and 0.84. Joslin (1923a, p. 167) calculates an average of 0.83 with 236 out of 239 normal individuals in the Harris and Benedict series (1919). He found also that with men of equal height variations of 14 kgm. in weight had no influence on the quotient. Magnus-Levy (cited by Du Bois, 1927, p. 72) has pointed out that 12 to 18 per cent of the basal calories are derived from protein, and it is customary to assume a mean of 15 per cent. With an average quotient of 0.82 the remaining 85 per cent of the calories are apportioned between fat and carbohydrate in the ratio shown in table 1. Of every 100 calories, therefore, approximately 15 derive from protein, 51 from fat and 34 from carbohydrate. This means that under basal conditions about half of the total calories are derived from fat.

The above serves for orientation in the following discussion of the influence of various factors on the respiratory quotient. It is not permissible in experiments, however, to assume a normal value; this must be determined in each case. It is evident, furthermore, that the basal quotient is not representative of the twenty-four hour catabolism, since it is measured at the end of the longest fast of the day.

Age has little influence on the basal respiratory quotient, after the first week of life. Of the metabolism of the fetus little is known, beyond that fact that histological examination has suggested an unusual supply of glycogen. Further, Lochhead and Cramer (1908) found that fetal rabbits near term have a considerable store of glycogen. This means nothing, however, as to oxidation. More direct evidence is con-

tained in the statement of Bohr (1900), that the guinea-pig embryo has a carbohydrate quotient. This statement requires confirmation. Data relating to the human fetus were obtained by Hasselbalch (1904) who made accurate measurements of the respiratory quotients of new-born infants and found them as high as unity. He concluded, in part, that the fetus has a carbohydrate metabolism. Benedict and Talbot (1915) made a much more extended series of observations, which did not, however, include quite such an early period in life. Though confirming Hasselbalch in most instances, they failed to find quotients approaching unity, and suggested that some of those observed by him might have been

TABLE 2

Respiratory quotients, calculated from the chemical equations for oxidation

Protein*	0.8016
Fat	0.707
Glycerol	0.86
Glyceric aldehyde	1.0
Betahydroxybutyric acid	0.89
Acetoacetic acid	1.0
Acetone	0.75
Carbohydrate	1.0
Lactic acid	1.0
Dihydroxyacetone	1.0
Pyruvic acid	1.2
Acetaldehyde	0.80
Methyl glyoxal	1.0
Glycuronic acid	1.2
Alcohol	0.667

* Allowing for end-products excreted in urine and stool.

unduly elevated by the muscular activity of the infant (see section 3). In general, the results of Benedict and Talbot were not such as to suggest an unusual supply of glycogen in the fetus. In the earlier hours of life the oxidation of carbohydrate was proportionately more rapid than in the adult, but the supply was soon exhausted. This was shown by the respiratory quotient, which was above 0.80, the adult basal level, in the earlier hours and below 0.80 later. Owing to the partial starvation incident to breast feeding it declined to 0.73 by the third day, showing the virtual exhaustion of available glycogen, less than 10 per cent of the calories being derived from carbohydrate. With the onset of

lactation a rise was observed, reaching 0.80, the adult figure, within the week.

The work of Bailey and Murlin (1915) included the study of 2 infants 6 hours old. The quotients were 1.12 and 0.85 respectively. Observations taken during the subsequent hours of life, showed, in agreement with Benedict and Talbot, a rapid exhaustion of the glycogen supplies.

After the vicissitudes of the first week the quotient reaches the adult level, as has been stated. Much work on young children has been done following the ingestion of milk. In this case the quotient tends to lie between 0.85 and 0.90 (Murlin and Hoobler 1915), but cannot be considered basal. Talbot (1917) obtained average quotients of 0.83 and 0.87 in prolonged observations of 2 breast-fed infants. In children from 6½ to 11 years, according to table 2 of Levine and Wilson (1926) the basal quotient averaged 0.80 or the same as in the adult. Much the same applies to the boys of 12 and 14 years, who had an average of 0.84, as shown in the work of Olmstead, Barr and Du Bois (1918). Girls 12 to 17 years of age had slightly lower quotients, according to Benedict and Hendry (1921). They were studied in groups of 12, and yielded respiratory quotients of 0.81, 0.81, 0.78 and 0.79. The observations were not strictly basal, as they began 7 to 8 hours after the last meal. In adult life, no change occurs, even in men above 75 years of age (Aub and Du Bois, 1917).

5. THE RATE OF UTILIZATION OF FOODSTUFFS. The general effect of the foodstuffs on the respiratory quotient can be predicted from the quotient of the food element in question. As already stated, carbohydrate has a quotient of unity and fat, 0.71. The former would tend to increase the quotient, the latter to lower it. The quotient may therefore be used to measure the rate at which the energy of fat and carbohydrate becomes available to the organism.

It has been repeatedly demonstrated that the most rapidly utilized of all the food materials are the sugars. Observations on the effect of these substances in man have been made by Zuntz and v. Mering (1883), Magnus-Levy (1894), Falloise and Du Bois (1896), Gephart and Du Bois (1915), (1916) and Benedict and Carpenter (1918). In the work last cited, the respiratory quotient was found on the average to rise after the first 20 minutes following the ingestion of 100 grams of glucose, to reach a maximum of about 0.90 in one to one and one-half hours, and to remain above the normal level for 6 to 7 hours. With the ingestion of the same quantity of levulose or sucrose there was an immediate rise to unity, or even above, but the effect was not substained so long. Lactose

was similar to dextrose, although the action was not so marked. With larger quantities of dextrose, the quotient may remain near the maximum for at least 6 hours, as shown in the data of Gephart and Du Bois (1915).

The influence of starch has been studied by Magnus-Levy (1894) and by Amar (1912). Since it is converted into monosaccharides before use, the effect should be the same, except for the time necessary for hydrolysis. Accordingly, it is found that the quotient rises gradually, hour by hour, and maintains the rise for a longer time.

Since the quotient of fat is nearer basal than is that of carbohydrate, the effect is less easily demonstrated. Magnus-Levy (1894) gave large amounts of bacon to dogs and noted that the quotient fell to 0.71 in 2 or 3 hours at the earliest. The effect continued 24 hours. Falloise and Du Bois (1896) found abnormally low quotients after a meal predominating in fat. Gephart and Du Bois (1916), on the other hand, found an average quotient of 0.83 in the second to fifth hours after the taking of 79 grams of olive-oil. Neither do the figures published by Benedict and Carpenter (1918) give evidence of any consistent depression of the respiratory quotient after the ingestion of fats. These negative results may be due to the limitations in the quantity which the human being can ingest. Murlin and Lusk (1915) were able repeatedly to give 100 grams of a 75 per cent emulsion of fat to dogs and obtained a remarkably consistent effect on the respiratory quotient, with an average fall of 0.05. This drop usually occurred at least as soon as the second hour after the ingestion, and the depression was maintained at a constant level as long as observed, up to 10 hours. The heat production rose at about the same rate as the quotient, reached a maximum at 6 hours and fell to basal only after 9 hours. This increase was shown to be entirely at the expense of fat. Benedict and Carpenter (1918) found an increase in metabolism beginning in the first two hours, and sometimes as early as the first hour. On the whole the action of fat on the oxidation appears to be less abrupt than that of carbohydrate, but more sustained.

Since protein has nearly the same quotient as that of man under basal conditions, its effect cannot be demonstrated by this means. The increase in the nitrogen excretion is demonstrable in about an hour, and is maximum in about 6 hours, both in man (Aub and Du Bois 1917b) and in the dog (Williams, Riche and Lusk, 1912). With the large quantities of meat which this animal can ingest, the effect may last 24 hours. Other evidences of the action of protein are the heat production, the

sulphur excretion, and in addition the elimination of protein sugar in phlorhizin glycosuria. All of these respond more sharply to the ingestion of protein than does the nitrogen excretion, as stated by Aub and Du Bois (1917b). According to their discussion this food is therefore available for oxidation within 2 hours after ingestion. This is a generous estimate, as indicated by the fact that in the dog nearly the maximal effect on the nitrogen metabolism is reached in $\frac{3}{4}$ to $1\frac{1}{2}$ hour after the meat. In phlorhizin glycosuria the sugar from the ingested aminoacids is excreted as rapidly as glucose itself. The slight delay in the utilization of protein is therefore not so much a question of metabolism as of absorption.

The three classes of foodstuffs differ, therefore, in the rate at which they become available. Sugar is the most promptly oxidized after ingestion, and reaches the maximum first. With fat and protein there is a greater initial lag, and more time is required to obtain the maximum effect.

The reason why the basal respiratory quotient is not a fixed quantity is implied in the above description of the effect of carbohydrate. Large amounts of this food have an effect even more prolonged than has been indicated. Thus by giving as much as 300 and even 500 grams with the evening meal, Benedict, Emmes and Riche (1911) were able to observe basal quotients averaging 0.86 to 0.90, and Krogh and Lindhard (1920) were able by the same means to produce similar effects. On the other hand, when carbohydrate is missing from the diet, the stores of glycogen are gradually consumed, and the basal quotient approaches the level of protein-fat combustion, or about 0.71. Examples of this may be found in the work of Benedict and Higgins (1912). These are the reasons why the basal respiratory quotient cannot be assumed, but must be determined in each case.

6. THE EFFECT OF FASTING. Much has been written on this subject, and accounts of it, with the literature, will be found in the works of Lusk (1919) Du Bois, (1927) and Grafe (1923). As stated above, the metabolism fourteen hours after the last meal varies between wide limits, averaging about 15 per cent of protein, 51 per cent of fat and 34 per cent of carbohydrate. The celebrated work by Benedict (1915) with Levanzin as the subject may serve as an example of the further course of a fast. By the end of a week the respiratory quotient was reduced to a level which in conjunction with the nitrogen of the urine showed the derivation of only 1 to 4 per cent of the total calories from carbohydrate. The oxidation of this substance continued at about the same rate but

ceased by the end of the second week. The available stores of glycogen were therefore exhausted in a comparatively short time, although the subject was on a normal diet before the fast began. For the remainder of the 31 days without food only protein and fat were consumed, 14 to 19 per cent consisting of the former. To judge from experiments on animals, the body fat would gradually be used up, and more and more of the burden would fall on protein. This effect was observed by Loewy on himself as a result, not of complete starvation, but of undernutrition prolonged to three years (Lusk, 1921, p. 535).

THE STORAGE OF GLYCOGEN. The quantity of glycogen in animals may be determined after death by direct chemical examination. Few such determinations have been performed in man, since it is rare that the material is fresh enough. Indirect measurements by means of the respiratory exchange have been made, however, the earliest example being the work of Benedict (1907), who made determinations based in part on the carbon of the urine. More recent estimates have been based entirely on the respiratory exchange and urinary nitrogen, as described earlier in this paper. The principle is simply to determine the total quantity of carbohydrate oxidized after the supply has been cut off. When 24 hour measurements of the respiratory exchange are not practicable determinations may be made at 12 to 24 hour intervals, and the carbohydrate oxidized per hour plotted as ordinates against the time in hours. The area under the curve then gives the total combustion of this substance. The first determination is made at some arbitrary time when the effects of the last meal are supposed to have worn off. This method gives, not the total glycogen content of the organism, but that portion of it which can be oxidized under ordinary conditions. For complete removal, to judge from experiments on animals, the use of phlorizin, adrenalin, shivering, and even strychnine would be required. Details of the method and the assumptions upon which it rests have been discussed by Richardson, Levine and Du Bois (1926).

For comparative results the subjects should be under the same conditions of diet and exertion, and the most practicable way of attaining this is to restrict the exercise and supply an excess of carbohydrate in the diet. The latter precaution was taken by Schoendorff (1903), who found the glycogen in the liver of dogs to vary from 4 to 19 per cent. Benedict (1907) found the same variability in healthy young adults on an unrestricted diet. In striking contrast to the foregoing is the extreme constancy found by Levine, Wilson and Rivkin (1926) who studied the glycogen content of five children, two of whom were normal,

one obese, one subject to cyclic vomiting, and one diabetic. Both the diet and the physical exertion were carefully controlled, and on a basis of square meters of body surface the variations, excluding the diabetic, were only from 192 to 201 grams. In dogs Fisher and Lackey (1925) found that the quantity of glycogen stored was almost constant for each tissue on a similar diet. These results could be reconciled with the lack of constancy found in earlier work above mentioned, on the theory that each individual has a storehouse of glycogen of maximum capacity, at times filled and at times partially empty.

However this may be, the results serve to give an idea of the total quantity of readily oxidizable glycogen present in man. Benedict's earlier (1907) observations gave from 93 to 232 grams per man (Du Bois 1927). Calculations from the work on the fasting subject L. (Benedict, 1915) yield 160 grams, about the same as the average of the earlier work. This subject was not on a measured diet before the fast, but partook of food containing an abundance of carbohydrate. A healthy young woman observed by Joslin (1923a) oxidized 370 grams of carbohydrate in a 4-day fast. The children studied by Levine, Wilson and Rivkin (1926) averaged 186 grams each.

The chief diseases in which the glycogen content has been of interest are diabetes and exophthalmic goitre. Few observations are extant in human diabetes, but it is known that in the experimental form glycogen disappears from the liver (Minkowski, 1893; Macleod, 1922; Fisher and Lackey, 1925). Post-mortem examinations in man have yielded considerable quantities (Kulz, 1876; v. Mering, 1877) and puncture of the liver of diabetics has demonstrated the presence of glycogen, according to Joslin (1923b). The latter describes a patient who, to judge from clinical evidence, was able to store 520 grams of carbohydrate in the course of an oatmeal "cure." Rabinowitch (1925) observed that both the blood-sugar and the respiratory quotient might remain unaffected by the ingestion of 100 grams of glucose, and concluded that "in a mild diabetic in a state of starvation, the storage mechanism may be unimpaired, or at least not impaired enough to be demonstrated by methods now in use." Richardson and Levine (unpublished data) using the method outlined above, found a normal supply of 386 grams in a mild diabetic. In spite of a moderately severe diabetes two patients studied earlier by Richardson and Ladd (1924) had quotients such as to indicate the oxidation of 43 and 81 grams respectively of carbohydrate in a two-day fast. In the work already mentioned, Levine, Wilson and Rivkin (1926) determined the respiratory exchange of a diabetic child

who had clinically a tolerance for 60 grams of carbohydrate and found the glycogen stores to be about two-thirds of the normal. On the whole the evidence shows that the defect in the storage of glycogen is relative and not absolute, and is dependent on the severity of the disease.

The storage of glycogen in exopthalmic goitre is a subject on which considerable investigation has been done. As is well known, the clinical tolerance for glucose is diminished in this disease; that is to say, a higher and more sustained rise of blood-sugar is observed after the ingestion of this substance than is found in normal individuals. This suggested a defect in oxidation of carbohydrate, but the respiratory quotient in two observations by Du Bois (1916) failed to confirm this idea. His results were substantiated by Sanger and Hun (1922) who obtained simultaneous curves of the respiratory exchange and blood-sugar in a larger series of patients. They concluded as Du Bois had done, that there was no defect in oxidation but rather an abnormality in the mobilization of glucose. Sanger and Hun interpreted their findings, i.e., the heightened blood-sugar curve and the normal oxidation of carbohydrate, as showing a defect in the mechanism by which glycogen is stored. In support of this theory, they cited the well-established fact that in animals the feeding of thyroid is followed by disappearance of glycogen. The authors who had demonstrated this fact are not, however, in agreement as to its cause, which might be ascribed, not to a defective storage of carbohydrate, but to increased oxidation. Parhon (1913) gave in part the latter explanation; Kuriyama (1917, 1918a, b) failed to commit himself definitely, one way or the other, while Cramer and Krause (1913) took the view that the storage of glycogen was defective. The last-named authors based their arguments mainly on the idea that excess of thyroid secretion decreases the oxidation of carbohydrate. The above-mentioned work of Du Bois (1916) and of Sanger and Hun (1922) subsequently showed this not to be true of exophthalmic goitre, and these results were confirmed in rats by Cramer and M'Call (1917, 1918), who found an actual increase in the oxidation of carbohydrate as a result of feeding thyroid. The latter maintain, however, that this is the effect and not the cause of the disappearance of glycogen from the liver. In other words, they postulate a primary defect in the mechanism of storage and a secondary increase in the oxidation of carbohydrate. They give no direct evidence, however, of this primary defect, and the work of Richardson, Levine and Du Bois (1926) on two cases of exophthalmic goitre tends to show that it does not exist. After an ample diet rich in carbohydrate both of the patients were found

to have glycogen stores at least equal to the normal individual with whom they were compared. No defect was found, or at least too trifling a one to resist the mass action of a diet rich in carbohydrate. Should these results be confirmed for exophthalmic goitre in general they would argue against a defect in the mechanism by which glycogen is stored.

Mention should be made of the alarming ketosis which developes in these patients after only 48 hours of fast. This procedure is actually dangerous, except under the closest observation.

7. THE SOURCE OF THE ENERGY USED FOR MUSCULAR EXERTION. In discussing the sources of energy used in muscular exertion we may consider first the foodstuffs utilized in the long run, laying aside for the moment the question whether one or all three are concerned in the immediate chemical reaction which provides energy for the oxidative phase of muscular activity. As previously remarked, when oxidation is complete, the respiratory quotient is independent of the intermediary steps in the metabolism, and may therefore be employed to solve the problem at hand. Even without the use of the quotient, it is obvious from the consideration of the diet that no one of the major food materials is indispensable. The bulk of the food of physically active individuals, laborers and the like, is used to supply energy for muscular exertion and it is well known that the diet of such people varies enormously in different parts of the world. The Chinese coolie has often been contrasted with the Esquimo of the far North; the former is capable of enormous exertion on a diet which at best consists mainly of rice, while the latter depends upon flesh and fat. From diets such as these, one may conclude that the energy used in muscular work may be originally derived either from carbohydrate, or from other substances.

Passing to more definite data, one may first consider protein. In civilized life this substance has very little to do with muscular exertion, provided sufficient calories are available in other forms. Thus the ascent of a mountain (Fick and Wislecenus, 1889, cited by Lusk, 1919) or prolonged stationary work (Pettenkofer and Voit, 1866), has comparatively little influence on the nitrogenous metabolism. In v. Mueller's clinic the same was found to be true of prolonged walking; Kocher (1914) found little or no change in the excretion of nitrogen, whether the diet consisted of fat and carbohydrate or of fat and protein. The exercise, though sufficient to double the expenditure of energy, raised the excretion of nitrogen in some instances not at all, and at most 7.9 grams or 209 calories. Since the increase in total metabolism was 2483 calories the maximum share of protein in the work was 8.4 per

cent. Krummacher (1896) found that the increase in the elimination of nitrogen could account in three experiments for only 25, 15 and 3 per cent of the external work. Since the expenditure of heat must have been some 5 times as great as the external work, the share of protein was approximately 5, 3 and 0.6 per cent. The data of Cathcart and Burnett (1926) yield a much higher percentage, 12, 25, 7 and 30 per cent in four groups of experiments, as calculated by the reviewer. Since the exercise was short, this effect may have been a temporary one, such as the removal of "deposit" protein suggested by Chambers and Milhorat (1927).

The chief and sometimes the entire burden of exercise falls then upon carbohydrate and fat. It is distributed according to the composition of the diet on the days preceding the exercise. The more carbohydrate eaten the greater the quantity oxidized, and the higher the respiratory quotient (Frentzel and Reach, 1901), even if the work be performed, as it usually is, without breakfast. In the observations of Benedict and Cathcart (1913) the average was 0.90, as compared to 0.82 after a diet lacking in carbohydrate. Very similar results in this respect were obtained by Krogh and Lindhard (1920). Furusawa (1925) calculated the respiratory quotient for the extra metabolism of work, and found it to be unity. It is evident that a large percentage or even the whole of the energy for muscular work may come from carbohydrate.

That this food is not indispensable as a fuel may be inferred from the low quotients observed after carbohydrate starvation by Krogh and Lindhard (1920) and in one case by Henderson and Haggard (1925). Many of these are near the level for combustion of fat. Diabetics, who can utilize but little carbohydrate, were shown by Grafe and Salomon (1922) and Richardson and Levine (1925) to react to exercise with quotients which imply a large preponderance of fat. In Hill's laboratory Hetzel and Long (1926) calculated in diabetic individuals the respiratory quotient for the excess metabolism of exercise, and found it to be low. These observations signify that in muscular work the oxidation of fat may predominate to the almost complete exclusion of carbohydrate.

It is evident from the preceding paragraphs that muscular work can derive its energy in part at least from each of the three food materials. The next question to be considered, is whether these can be oxidized in their original state, or only after conversion into carbohydrate. The argument may well start from the physiology of muscle, as developed by Emden and his school, by the English physiologists, and by Meyerhof. This work has been frequently reviewed, for instance by Hill,

(1924), Hill and Meyerhof (1923), and Atzler (1927). Largely as a result of these investigations it is known that the actual contraction of muscle is energized by the transformation of glycogen via hexose phosphate into lactic acid. No oxygen is needed for this process. During recovery, however, the lactic acid is reconverted into glycogen, a compound of higher potential energy, and for this the oxidation of some foodstuff is required. Theoretically, anyone of the three may serve, as may become clearer from the statement of Hill, Long and Lupton (1924a).

> It is possible that the recovery process is really of the nature of a "coupled reaction," in which the restoration of lactic acid to glycogen occurs as part of a definite chain of chemical processes in which oxygen is used and foodstuff oxidized to provide the necessary energy. It is equally possible, however, that the restoration of lactic acid to glycogen occurs in a manner analogous to the recharging of an accumulator; there sulphuric acid is restored from the lead plates to the solution around them by means of some mechanism (*e.g.*, a dynamo) entirely separate from the accumulator, driven by a separate supply of energy, and merely providing the power to effect the restoration.

In other words, the recharging of muscle might be done by a separate source of power, using fat as readily as carbohydrate. The theory that the latter alone can serve the recovery process in muscle, is now prevalent, but according to the above quotation it is not inherent in the mechanism of muscular activity. Meyerhof (1925) does not insist upon it.

At the outset it is evident that, if carbohydrate is the sole source of energy for the immediate reaction, and if in the long run fat is utilized as well, fat must be converted into carbohydrate. Once the premise is accepted, the conclusion follows without further evidence. From this point of view, the calculation of Boothby and Sandiford (1924) is superfluous, although it was advanced in support of a possible conversion of fat into carbohydrate-like substances. In the case of a diabetic patient, they computed that the total quantity of carbohydrate oxidized was insufficient to supply energy even for the very moderate needs of hospital existence. There is nothing in the theory of muscular physiology however, which demands the *oxidation* of any carbohydrate whatever. The conception that the recovery process requires this substance and no other, is based on evidence, a review of which is to follow.

This evidence concerns, first, the respiratory exchange and chemical alterations in excised muscle; second, the respiratory quotient for the extra metabolism of exercise in man, and third, certain aspects of the

quotient during exercise, together with the relative efficiency of foodstuffs. In excised muscle experiments have failed until very recently to prove the oxidation of fat. Meyerhof (1919a) studied the excised muscle of the frog, using the fatigued gastrocnemius in the process of recovery. He found the respiratory quotient to average 1.02, implying the oxidation of carbohydrate alone. Winfield (1914–15) was unable by chemical methods to demonstrate the oxidation of fat, although the negative value of this evidence is open to question. Meyerhof and Himwich (1924) studied the respiratory quotient of excised diaphragmatic muscle of the fat-fed rat. They found no sure indication that fat could be burned instead of carbohydrate, nor any rigorous proof that it could not. Granting the validity of the foregoing results, they may signify, not that carbohydrate is the only possible food, but simply that it was the only one available under the conditions of the experiment. That fat can be oxidized by excised tissue has been demonstrated in Meyerhof's laboratory by Takane (1926) who determined the respiratory quotients and complete carbohydrate balances on the diaphragm and liver of the rat. With muscle he found that the disappearance of carbohydrate could account for 20 to 50 per cent of the oxygen consumed. Disappearance of lactic acid accounted for 15 per cent more. Since, to judge from the formation of ammonia, the diaphragm derived only 10 per cent of its calories from protein, the remainder of the oxygen must have been utilized in the oxidation of fat. This work is not inconsistent with recent observations on mammalian muscle in situ. Himwich and Castle (1926) were able to isolate a muscle of the dog without interrupting the normal circulation. By analysis of the arterial and venous blood the respiratory quotient was determined, the average for resting muscle being 0.71 ± 0.10. Himwich and Rose (1927) used the same procedure with exercising muscles and obtained an average quotient of $0.80 + 0.05$. These results imply the oxidation in the muscle itself of a considerable proportion of fat, either directly, or by way of carbohydrate.

If the foregoing results be taken at their face value they suggest that muscle oxidizes fat under certain conditions, and not in others. That it is not a mere matter of technique is indicated by the fact that the same authors have figured in experiments of both types. If on the other hand the differences were merely technical the conflict would be too great to afford any experimental basis for the theory that muscle can subsist only on carbohydrate.

Further evidence in favor of the latter theory was obtained in Hill's

laboratory by Furusawa (1925) who measured the respiratory quotient for the extra metabolism due to exercise in man, and found it to be unity, provided that the exercise is brief. He believes that only under these conditions is it possible to avoid disturbances in that portion of the metabolism which is not concerned in the exercise. For longer period the quotient is admittedly much less than unity, as has been confirmed by the Sage investigators (Richardson and Levine, 1925) and by Wilson, Levine, Rivkin, and Berliner (1927), but here it may be the resultant of two processes, the conversion of fat into carbohydrate with the production of a low quotient, and the oxidation of carbohydrate, of which the quotient is unity. For evidence of the oxidation of fat in muscle one might reasonably look to diabetic patients. These differ, in fact, from normal individuals, in that, in the absence of insulin, the respiratory quotient for the excess metabolism of exercise is low, even for short periods of exertion (Hetzel and Long, 1926). Although these findings give no additional support for the idea of the exclusive use of carbohydrate by muscle, they are interpreted by the authors in terms of this theory. Furusawa's (1925) results failed of confirmation at the hands of Henderson and Haggard (1925), in so far as the two sets of observations are comparable. Although the exercise was perhaps too heavy to conform to the conditions laid down by Furusawa the results are of great interest. The respiratory quotient during 3 to 5 minutes of rowing plus 10 to 15 minutes of rest was compared to the basal. In only one experiment out of seven was there a definite increase as the result of exertion, and in two there was a definite drop. Particularly striking was the individual with whom the quotient for exercise plus rest was 0.73, as compared to a basal or 0.75. The evidence obtained from man under varying conditions of exercise is therefore contradictory. Even if it were not, a quotient of unity need not mean that carbohydrate is the only possible fuel. It may signify merely that carbohydrate was the substance most available at the time.

Further data on the source of the energy used in muscular work was obtained by Krogh and Lindhard (1920) in a very careful study of the effect of exercise in normal individuals. Their results led them to the tentative theory that fat might be converted into carbohydrate or the reverse. This was based in part on the observation that quotients at the extreme normal range during rest, tended to assume intermediate values with exercise. Their explanation was that the resting quotient was unduly elevated by the laying down of fat which originated from carbohydrate and attained the true value only with exercise. The reverse

process could be used to explain the behavior of the low quotients. They found also, in agreement with Anderson and Lusk (1917), and with Cathcart and Burnett (1926), that fat is a less efficient fuel than carbohydrate for mammalian muscle. This they thought due to the loss of energy incident to the conversion of fat into carbohydrate. A similar explanation was given for their observations on the basal metabolism. Boothby and Baborka (1924) have failed in part to confirm their first point, which can, moreover, be quite as well explained as the result of a qualitative change in the metabolism. The relatively low efficiency of the human machine on a fat diet may be due to some peculiarity of the machine itself, rather than to the loss of energy incident to the conversion of fat into carbohydrate.

On the whole the evidence in favor of the exclusive oxidation of carbohydrate by muscle is balanced or outweighed by other investigations. Experimental basis for the conversion of fat into carbohydrate is therefore lacking. On the other hand, observations with the phlorhizinized dog are difficult to reconcile with this idea. When the drug is given to this animal, sugar appears in the urine, and after a few days reaches a constant level at the rate of 3.65 grams of dextrose for every gram of nitrogen in the urine, as demonstrated by Reilly, Nolan and Lusk (1898) and by Stiles and Lusk (1903). When glucose is given to an animal in this state the D:N ratio rises, and the increase in sugar accounts for a large proportion of the quantity ingested. (Stiles and Lusk, 1903.) This fact has been abundantly confirmed. On the other hand procedures which stimulate the metabolism of fat have no effect on the ratio, whether the increase be accomplished by feeding (Moritz and Prausnitz, 1890) (Lusk, 1901) (Wierzuchowski, 1927) (Reilly, Nolan, and Lusk, 1898) or by exercise (Lusk, 1908). If sugar were formed from fat, the greater part should be excreted, as in the case with ingested sugar. The evidence is therefore against this conversion.

This point of view was elaborated by Richardson and Levine (1925b). Since then, work by Wierzuchowski (1926), and Deuel, Wilson and Milhorat (1927) has shown that in phlorhizin poisoning the defect in the utilization of ingested sugar is not necessarily complete, and that therefore a certain amount of sugar could arise from fat and escape detection in the urine. If this were the case, it would be necessary to explain why most of the ingested sugar is excreted, and none of that arising from fat.

On the whole the weight of evidence is against the formation of carbohydrate from fat. The decisive experiment on this question is yet to be

performed. Meanwhile, it is justifiable to accept the respiratory quotient at its face value, during exercise, as at rest.

8. THE RESPIRATORY QUOTIENT IN DIABETES. Diabetes is characterized primarily by waste of carbohydrate, which is excreted as glucose in the urine, and secondarily by loss of weight, increase in the metabolism of protein, and the accumulation and excretion of keto-acids and acetone. As first suggested by Minkowski (1893, 1911) the essential defect of the disease is that carbohydrate is not oxidized. Among the evidences of this defect is the fact that the basal respiratory quotient is depressed in this disease. There is abundant evidence in the literature that this is the case, as demonstrated for example by Falta, Grote and Staehelin (1907), Verzár (1912), Verzár and v. Fejer (1913), Verzár (1914) and Hédon (1926) in experimental pancreatic diabetes; and by Allen and Du Bois (1916), Joslin (1923a) and Dautrebande (1926) for the human. A patient exhaustively studied by Wilder, Boothby, and Beeler (1922) had an average basal respiratory quotient of 0.693. Instances of similar values might be multiplied at will. Further evidence of the nature of diabetes is the fact that the elevation of the quotient observed in normal individuals after the ingestion of sugar is lacking in the more severe forms of the disease (Joslin, 1923a, p. 249; Richardson and Mason, 1923, p. 599; Wilder, Boothby and Beeler, 1922, p. 331; Rabinowitch, 1925). Exceptions have been noted by McCann and Hannon (1923). Failure of carbohydrate in general to elevate the respiratory quotient was noted by Nehring and Schmoll (1896–97) Rolly (1912) Bernstein and Falta (1918). There is therefore no doubt but that the respiratory quotient is depressed in diabetes even after the ingestion of carbohydrate, and this has been justly interpreted to mean that the oxidation of carbohydrate is defective.

Were this substance alone affected, the interpretation of the quotient in terms of the foodstuffs oxidized would be a simple matter, and the calculations described in section 1 of this paper would require no correction. The non-protein quotient would be diminished in proportion to the severity of the disease, until in the worst cases it would be 0.71, the respiratory quotient for fat.

As a matter of fact in severe cases other food-constituents are affected as well, and this tends to make the quotient even lower. The faulty oxidation of protein has been extensively studied in phlorhizin glycosuria, notably by Lusk, as cited in section 7. In this condition glucose continues to be excreted in the urine when no carbohydrate is accessible either in the diet or in storage in the body. The excretion is then pro-

Theoretically speaking, the loss of calories in the urine should be taken into account in the interpretation of the reduced quotient, but this may be neglected, as it is less than the experimental error.

Of greater importance is the correction for the acid effect of the ketones. In the process of formation these act like other non-volatile acids in that they expel carbon-dioxide from the body, thereby raising the quotient above the true or metabolic value. This action depends upon the rate of their accumulation in the body, and the manner of their neutralization, whether by sodium bicarbonate or ammonia (Lusk, 1915b). To correct for this factor, a knowledge of the carbon-dioxide content of the entire body at the beginning and end of the observation is required. So far as the blood is concerned this factor may be quantitated by means of the carbon-dioxide combining-power, but this does not necessarily reflect the conditions in the tissues. Moreover, the total volume of tissue subject to change in this respect is unknown, and therefore the net change in carbon-dioxide content cannot be calculated. A limited amount of reliance may be placed on the fact that the two effects of the keto-acids tend to neutralize each other. Where quantitative interpretation of the quotient is desired, however, it is best to select conditions under which the formation of ketones is equalled by the removal, as shown by constancy of the carbon-dioxide combining power of the blood. Better still is to confine the observations to diabetes of a moderate degree of ketosis.

The foregoing discussion of the respiratory quotient in diabetes demonstrates the difficulty of calculating the exact quantities of protein, fat and carbohydrate oxidized. It also has a direct bearing on the theory of the disease, in that the quotient may be considerably below 0.71, the value for fat combustion, without implying any process other than oxidation.

That the respiratory quotient is not necessarily low, even in severe diabetes, has been brought out by Joslin (1923a) in a continuation of the work of Benedict and Joslin. He reports occasional values far above the normal basal level. These were observed in emaciated patients with depressed metabolism and subnormal weight, without acidosis, but as a rule *in extremis*. The highest quotient of 0.94 was recorded in a diabetic whose basal metabolism was −37 per cent and whose weight was 50 per cent below normal. This group contrasts with those who had very low quotients, relatively high metabolism, and severe acidosis. The last may have been a factor in the case of the low quotients. Joslin emphasizes the relation between high quotients

and depressed metabolism, but does not stress the simple arithmetic relation between the two. When a given quantity of carbohydrate is oxidized, the lower the total caloric expenditure the higher the respiratory quotient, since the latter is a ratio and not an absolute number. If for instance, in Joslin's case 1011 the total metabolism were to increase from −37 per cent to normal by an increase in the metabolism of fat, the respiratory quotient would fall from the observed value of 0.94 to 0.85. Since other explanations appeared inadequate, Joslin has suggested that the high quotients may be due to the conversion of carbohydrate into fat. This occurs normally, and would not be surprising in the diabetic. It has been pointed out above, however, that the conversion of one material into another can affect the respiratory quotient only when the product is laid down in the body rather than oxidized. A continuously high respiratory quotient from this cause would imply fattening rather than emaciation. A similar patient with a high basal respiratory quotient was observed by Richardson and Mason (1923) and at autopsy scarcely a trace of fat could be found. According to this reasoning, the product of the conversion cannot be fat, but might be some other material of unknown composition and low respiratory quotient. In any case the objection does not apply to Joslin's numerous experiments on levulose, ingestion of which was followed by a rise in the respiratory quotient reaching unity in two cases, both severe.

Parallel results have been obtained by Hédon (1926) with the depancreatized dog. The respiratory quotient reached the fat level after the fourth day, and gradually rose, in one case to 0.84 just before the death of the animal. Two other instances in man have been reported by Holten (1924) who has added the interesting finding that insulin reduces the respiratory quotient of such patients in contradistinction to its usual effect in diabetes. He suggests that this is caused by the cessation of the conversion of carbohydrate into fat. An alternative explanation is that when the glycogen stores are low insulin diverts sugar from oxidation to synthesis, thereby reducing the quotient.

In summary, the respiratory quotient is low in severe diabetes. The exact interpretation is difficult, owing to the secondary effects on the oxidation of protein and fat. The depression of the quotient is not universal, and abnormally high figures have been observed, particularly in severe diabetes associated with emaciation. These figures may be explained, in part at least, without invoking the conversion of carbohydrate into fat.

9. THEORIES OF DIABETES. In the foregoing discussion it is assumed that diabetes is due to a defect in the oxidation of carbohydrate. An alternative theory is current, especially on the continent of Europe, namely, that combined with a normal oxidation there is an overproduction of glucose. If the latter is true, the newly formed glucose must have its source in protein or fat. Of these only the origin from fat is a matter of controversy, and the evidence in its favor has been criticized above in the section of muscular exercise. This related, however, only to the normal organism, and the supposed neoformation might be confined to diabetes.

Experiments on this subject are of two general types; those designed to demonstrate the oxidation of carbohydrate in isolated organs of the diabetic animal, and those intended to prove a new production of sugar. In the former the respiratory exchange may be measured either direct, or from alterations in the gaseous content of the nutrient fluid (blood or its substitute) in its passage through the tissues. In the second type of experiment changes in the carbohydrate content of the tissues or nutrient fluid are noted, usually the reducing power of the perfusate.

From the welter of conflicting evidence obtained by the above means one is led to suspect sources of error, and these, with the aid of recent discoveries, are not hard to find. The precautions required for obtaining an accurate respiratory quotient which is at the same time representative of the metabolism have been described at the beginning of this paper. They apply with redoubled force to experiments on any portion of the body which has been subjected to interference with its oxygen supply, or to other unphysiological conditions. Many tissues have the power of converting glucose into lactic acid, not only under anaerobic conditions, but normally as well. It has been known for some time that in muscle the carbohydrate passes from glycogen through intermediary carbohydrate to lactic acid and back again to glycogen. Other tissues, such as kidney, liver and gray matter, are capable of producing lactic acid, and the reverse change is suggested by the observation of Meyerhof and Lohman (1926) that lactate disappears from a solution in contact with slices of these organs. In isolated tissues, as in the whole organism, lactic acid expels carbon-dioxide, thereby causing an undue rise in the respiratory quotient. When lactate disappears, base is retained and the respiratory quotient is depressed.

That the changes in the carbon-dioxide content of the preparation must be allowed for is evident from these experiments. An example of the error caused by the neglect of these precautions is afforded by the

work of Porges (1910) and of Porges and Salomon (1910) which was much cited at one time in support of the overproduction theory of diabetes. These authors tied off the hepatic and abdominal vessels of depancreatized dogs and found that the respiratory quotient was unity. They concluded that in diabetes the oxidation of sugar is not disturbed. Murlin, Edelmann and Kramer (1913) were able to demonstrate that the changes in the quotient caused by this procedure have no relation to oxidation, and are, in fact, mainly due to alterations in the carbon-dioxide content of the preparation. The conclusions of Porges and Salomon were attacked on much the same ground by Verzár (1911) Rolly (1912) and Fischler and Grafe (1912) and are given little credence at present. Recently Markowitz (1928) obtained results similar to those of Porges and Salomon, but failed to meet the criticisms described above.

When the respiratory quotient of an organ is determined from changes in the gaseous content of the nutrient fluid, as in perfusion experiments, it is further necessary to make allowances for changes in water content of the fluid in its passage through the tissue.

Much work designed to show by chemical analysis the neogenesis of carbohydrate is of dubious value in the light of the newer knowledge of the transformation of carbohydrate. In many perfusion experiments sweeping conclusions have been drawn solely from changes in the reducing power of the perfusate. The events are much too complicated to permit this simple deduction. To prove the neoformation of carbohydrate, analyses are required, not only of glucose, but of glycogen, intermediary carbohydrate, and lactic acid as well; in other words, a complete carbohydrate balance. Since the events in the nutrient fluid need bear no quantitative relation to those in the tissue, the latter must also be included in the determinations. The authors who have perhaps come nearest to fulfilling these requirements are Burn and Marks (1926) who demonstrated in the perfusate of the liver of the normal fat-fed animal an increase in the reducing substance which they could not attribute either to protein or carbohydrate. One liver taken from a depancreatized cat gave a similar result. They did an almost complete carbohydrate balance, omitting only the lactic acid of the liver. Since, however, this organ was necessarily subjected to anaerobic conditions at some time in the 20 to 30 minutes required for preparation, the accumulation of lactic acid in the cells before the onset of the experiment was highly probable. With the beginning of perfusion by means of oxygenated fluid, the reconversion of lactic acid into glucose would be

expected. This might well have been the source of the increase in the glucose of the perfusate noted by these observers. The above serves to illustrate the hazards of perfusion experiments. A much more satisfactory technique is the Warburg-Barcroft, which permits the simultaneous analysis of tissue and nutrient fluid.

When the above criteria are applied to the literature, only a few experiments qualify. Most of them relate to the normal organism and not to the diabetic, and have been considered in the section on muscular exercise. Peserico (1925) determined the respiratory quotient of the isolated mammalian heart and found it to average 0.72 in depancreatized animals, or not far from the figures obtained with the intact diabetic organism.[1] This observation is exceptional in that the bicarbonate content of the nutrient fluid was measured before and after the experimental period. The author gives no figures but states that he found no noteworthy change, often none at all. Other authors who have paid attention to this point may have escaped the attention of the reviewer.

The theory of defective oxidation is based on three main experimental findings; first, that the respiratory quotient of the diabetic is at a level which precludes the direct oxidation of carbohydrates; second, that in severe diabetes, in contrast to the normal individuals, no rise in this quotient takes place as a result of the ingestion of carbohydrate; third, that in diabetes of maximum severity there is no relation between the excretion of glucose and the metabolism of fat such as would be expected if fat were converted into carbohydrate. The first two points have already been discussed in the preceding section, and the third under "muscular exercise" (section 7). In addition certain recent experiments on insulin should be stressed. It is universally conceded that diabetes, whatever its nature, is due to a defect in the production of insulin. The mechanism by which insulin acts therefore has a direct bearing on the nature of the disease. The experiments described below, especially those of Bissinger and Lesser (1926), Takane (1926) in Meyerhof's laboratory and Cori and Cori (1926, 1927), give evidence that insulin stimulates the oxidation of carbohydrate. It follows that diabetes does the opposite.

The overproduction theory has been supported in particular by Von Noorden (von Noorden and Isaac, 1927) and Geelmuyden (1923). Minkowski (1911) defends the theory of defective oxidation, which he first propounded; Grafe (1923) discusses both sides of the question, and attempts to combine both theories. The neoformation of glucose may

[1] See also Richardson, Loebel and Shorr, p. 125, addenda.

be inferred, if the following statements are true: first, that the respiratory quotient in diabetes of maximum severity is lower than can be accounted for, except on the assumption that fat is converted into carbohydrate; second, that more glucose is excreted than can be explained by the catabolism of protein; third, that there is evidence of oxidation or new-production of glucose in isolated organs or tissues. In addition, analogy might be drawn from the very abnormal quotients observed in hibernating animals.

Much discussion has been evoked over the point first mentioned, namely, whether or not the respiratory quotient is below the minimum level which can be attributed to oxidation alone. Since the laying down of fat at the expense of carbohydrate causes a respiratory quotient above unity, so the laying down (or excretion) of carbohydrate formed by the reverse process might be expected to depress the quotient below this minimum. The lowest quotient compatible with the theory of defective oxidation can be calculated. Since glucose and the keto-acids are held to escape oxidation, protein is practically untouched. Thus:

100 g. protein give	58	gm. glucose (D:N 3.65)
	17.7	gm. beta hydroxybutyric acid
	17	gm. nitrogen
100	93	

Judging from the work of Cremer (1902) and Lüthje (1904) the glycerol of the fat is not oxidized, but instead is converted into glucose. Shaffer (1921c) has calculated the respiratory quotient of a mixture of fats similar to that found in the human, and found it to be 0.698, making a slight allowance for the conversion of glycerol into glucose. This figure was obtained as follows:

The average fatty-acid considered was given a formula of $C_{17.7}H_{34}O_2$. In 100 mol. fat there are 100 mol. glycerol and 300 mol. fatty acid.

300 mol. $C_{17.7}H_{34}O_2$	7560 O_2	→ 5310 CO_2
To convert 100 mol. glycerol to 50 mol. glucose requires	50 O_2	
	7610 O_2	5310 CO_2

giving a respiratory quotient of 0.698.

In the present case the fat is burned only to the stage of beta-hydroxybutyric acid. Each mol. of fatty acid is assumed to give rise to one mol.

of beta-acid. 2 mol. of the latter require 9 mol. of O_2 and yield 8 mol. of CO_2

	7610 O_2	5310 CO_2
Subtract for 300 mol. beta-acid	1350 O_2	1200 CO_2
	6260 O_2	4110 CO_2

giving a respiratory quotient of 0.655.

If all of the keto-acid reacted with $NaHCO_3$ each mol. would drive off one mol. of CO_2.

	6260 O_2	4110 CO_2
Add for CO_2 driven off		300
	6260 O_2	4410 CO_2

giving a respiratory quotient of 0.704.

If the acid were neutralized by ammonia the latter would be diverted from urea formation, and for every 2 mol. of alkali neutralized 1 mol. of CO_2 would be released.

	6260 O_2	4110 CO_2
plus CO_2 released from urea formation		150 CO_2
	6260 O_2	4260 CO_2

giving a respiratory quotient of 0.68.

The extent of this process is considerable. The excretion of ammonia in severe acidosis may reach 5 grams in 24 hours or 4.3 above the normal of 0.7. This increase would neutralize 26 grams of beta-hydroxybutyric acid.

However neutralized, the effect of the acid would be to expel carbon-dioxide. The tendency of the body to compensate for the acidosis has the opposite effect, in that free acid is excreted in the urine, and the equivalent base is retained in the body, where it causes the retention of carbon-dioxide. The net effect of the acidosis is a resultant, as follows:

1 mol. CO_2 expelled;	1 mol. CO_2 retained
(a) for each mol. of keto-acid neutralized by sodium bicarbonate	for each mol. of free acid excreted in the urine
(b) for each 2 mol. neutralized by ammonia	

The minimum respiratory quotient in total diabetes, making allowance for acid effects, therefore lies between 0.655 and 0.704, and probably about midway between.

If diabetes is in fact a defect in the oxidation of carbohydrate, the range of the quotients in cases of maximum severity should be that just mentioned. Du Bois (1927) has made a table of "abnormally low" quotients, and even in this selection quotients below 0.65 are in a minority, the lowest basal value being 0.638. He has pointed out that under the best conditions an error of 0.02 is to be expected. Among the authors tabulated is Joslin, (1923a) who in a series of 113 diabetics, reports only 9 patients with a total of 18 basal respiratory quotients below 0.70. Of these the lowest was 0.65, and occurred only once; there were two instances each of 0.66 and 0.67, and the rest were 0.68 and 0.69. Another patient included as an afterthought had basal quotients of 0.63 to 0.66. A severe diabetic observed by Wilder, Boothby, and Beeler (1922) had a minimum quotient of 0.65, a maximum of 0.74, and an average of 0.693.

Considering the extent to which the respiratory metabolism in diabetes has been studied, quotients below the theoretical minimum are unusual and offer scant support for the theory of neoformation of carbohydrate.

An overproduction of sugar might be inferred from a dextrose-nitrogen ratio above the maximum level which can be attributed to the conversion of protein into glucose. A number of investigators have obtained such ratios. Since, however, the value is markedly influenced by the secretion of stored carbohydrate, as well as by the surreptitious ingestion of food, such determinations are not conclusive. There are a number of observations to show that the dextrose-nitrogen ratio tends to approach a well-defined constant value. Minkowski's (1893) value of 2.8 to 1 for the depancreatized dog has been abundantly confirmed as an average; likewise the ratio of 3.65 to 1 demonstrated by Reilly, Nolan and Lusk (1898) for the phlorhizinized dog. That the latter figure is applicable to human diabetes was demonstrated by Mandel and Lusk (1904). A number of observations confirming their findings in human diabetes have been tabulated by Du Bois (1927). As stated above (section 7) this ratio is increased by the feeding of substances of a carbohydrate nature, and not by increasing the metabolism of fat. From this it may be inferred that the formation of sugar from fat does not occur.

The last conclusion has been widely accepted in this country at least, but the question has been reopened by observations in Toronto on depancreatized dogs kept alive with insulin. Chaikoff, Macleod, Markowitz, and Simpson (1925) found that the D:N ratio failed to reach any constant value, and declined constantly in thin animals to far below

the level of 2.8. Chaikoff (1927) working in Macleod's laboratory, came to the same conclusion, and found also that the ratio varied according to the state of nutrition, being higher in case the animal was well fed. In his opinion "the results of the investigation support the view that glucose is derived from fatty-acid in the diabetic organism." It may be pointed out that only one of his three animals had a ratio appreciably above 2.8 for more than three days, and this suggests the obvious explanation that the high values were due to the breakdown and excretion of glycogen. Chaikoff estimates this factor by glycogen determinations of liver and muscles of one diabetic animal as compared to other depancreatized dogs in an earlier stage of insulin deprivation, and computes that the loss was insufficient to account for the high value of the D:N ratio in the dog mentioned. The question arises, as to whether the experimental animal was in the same state of nutrition and had the same loss of glycogen as the controls. Judgment on this point may well be suspended.

The main difficulty for Grafe (1923) in accepting the theory of the defective oxidation of carbohydrate in diabetes is the evidence that glucose disappears from isolated organs of the diabetic animals. As reiterated above, this evidence does not withstand the criticism which is imposed by the newer knowledge of the metabolism of carbohydrate.

The low respiratory quotients which have been observed in the hibernating animal have been explained by some authors as due to the formation of glycogen from fat. Grafe (1923) who has reviewed the literature, points out that chemical examination has failed to demonstrate any such increase in glycogen. He attributes the low level of the quotient to differences in solubility of gases at low temperatures. Both gases are of course more soluble in the cold, but carbon-dioxide relatively more so than oxygen. The depression of the quotient is of long duration, because the metabolism is reduced to 1 per cent of the normal. It is evident, therefore, that to prove the formation of carbohydrate from fat in the hibernating animal, the effect of differential solubility would have to be eliminated. It may be added that even then the results would not necessarily apply to man.

On the whole the theory of the defective oxidation of carbohydrate in diabetes has many experimental facts in its favor, and little compelling evidence against it.

10. THE RESPIRATORY QUOTIENT AND INSULIN. As hinted in the preceding section, insulin has a beneficial effect in diabetes, too well known to require comment. It reduces the waste of carbohydrate,

lowers the blood sugar, decreases the ketosis, and improves strength and nutrition. If diabetes is due to a defect in oxidation, and if it is ameliorated by insulin, it follows that the latter stimulates the oxidation of carbohydrate. Conversely, if insulin stimulates the oxidation of carbohydrate, it must be inferred that diabetes does the opposite. The independent demonstration of the mode of action of insulin should therefore be of great fundamental importance.

With few exceptions the administration of insulin is followed both in the normal and the diabetic organism by a rise in the respiratory quotient. This has been found true of the normal animal of several species: the rabbit (Krogh, 1923, 1925); dog or rabbit (Dickson, Eadie, Macleod and Pember, 1924); the dog (Boothby and Weiss, 1925); the cat (Taylor and Olmstead, 1926); the rat (Gabbe, 1924, Cori and Cori, 1926); the mouse (Lesser, 1924, and Bissinger and Lesser, 1926); and guinea pig (or rat) (Cammidge and Howard, 1924). Few observations are reported on the normal man, but Lyman, Nicholls and McCann (1923) found a rise in three individuals. In experimental phlorhizin poisoning M. Ringer (1923) noted a rise in the respiratory quotient after insulin, as did Gaebler and Murlin (1925). A similar rise was observed in human diabetes by Fitz, Murphy, and Grant (1922). Joslin, Gray and Root (1922) obtained the same results, provided that food was given along with the insulin. Wilder, Boothby, Baborka, Kitchen and Adams (1922) found a greater increase when insulin was given with glucose or fructose, than was the case with the sugar alone. The rise in the quotient was observed also by Lyman, Nichols and McCann (1925), Burgess and associates (1923), Kellaway and Hughes (1923) and Holten (1924).

That insulin causes a rise in the respiratory quotient can hardly be doubted, but the interpretation of this fact is not simple. The disturbances of metabolism which complicate matters in severe diabetes take place in the reverse direction when insulin is given. Likewise in mild diabetes or even the normal state the results may be hard to interpret. For some reason the injection of insulin is often followed by an increase in the lactic acid of the blood (Briggs, Koechig, Doisy and Weber, 1923) and this expels carbon-dioxide from the body. Although the work of Tolstoi, Loebel and Richardson (1924) on animals suggested that this effect occurred only when the blood-sugar is reduced by insulin below the normal level, it is at all events a factor with which to reckon. In most experiments where enough data are available, the expulsion of carbon-dioxide from the body can be discerned by an increase either in

the ventilation of the lungs, or in the fixed acid of the blood. This probably originates in muscular contraction, which can be prevented by the injection of curare. In general, the formation of acid elevates the respiratory quotient mainly by increasing the ventilation of the lungs, and this can be prevented by artificial respiration. Krogh (1923, 1925) and Taylor and Olmstead (1926) observed both of these precautions, and still were able to record a marked rise of the respiratory quotient following the administration of insulin. These observations increase the probability that insulin stimulated the oxidation of carbohydrate. Obviously, they cannot be repeated in humans and the appreciable increase in ventilation which was observed as a result of insulin by Kellaway and Hughes (1923) and Holten (1924) must have had some effect on the respiratory quotient, although a negligible one, according to the calculations of the latter.

One method of controlling this factor is to determine the fixed acid of the blood at the beginning and end of the experiment. Tolstoi, Loebel, Levine and Richardson (1924) analyzed the lactic acid of the blood before and after determining the respiratory exchange. With one patient a pronounced rise in the respiratory quotient was twice observed although there was no increase in the lactic acid in either instance. The latter was thereby eliminated as a cause of the increased quotients in these two observations, although some other acid might also have been a factor. Other observers have determined the alkaline reserve of the blood, which falls whenever there is a rise of non-volatile acid. Dickson and associates (1924) found in dogs an almost invariable drop, which, however, they did not consider significant. Boothby and Weiss (1925) likewise demonstrated in the dog a fall in the carbon-dioxide combining power, in fact, sufficient to cause an error of 0.07 in the quotient. Similar effects were observed in man. They concluded that no inference can be drawn from the respiratory quotient as to the nature of the metabolism after insulin.

For reasons stated above in section 8, the exact effect of a given change in the carbon-dioxide combining power of the blood cannot be calculated. Observations sufficiently prolonged to allow this to return to the pre-insulin level are eminently desirable, but are not available so far as is known to the reviewer. If this condition were fulfilled, the respiratory quotient for the entire period might reasonably be interpreted as the result of oxidation alone. Even this does not take into account the possibility of the formation of fat from cabohydrate in diabetics, and its cessation with the administration of insulin, which is suggested by an

interesting observation (Holten, 1924). This author studied two very emaciated diabetics whose basal respiratory quotients were above 0.90, and noted after insulin a fall in the quotient instead of a rise. His explanation is not, however, the only conceivable one, since a fall in the quotient would result if glucose were merely diverted from oxidation to be stored as glycogen. Polymerization of glucose is one of the established effects of the drug (Fisher and Lackey, 1925; Bissinger and Lesser, 1926; Macleod, 1922, 1926).

Observations on perfused organs require even more rigid control than in the case of the intact organism, where the conditions are more physiological. This has been explained in the discussion of diabetes (section 9). Peserico (1925) perfused the beating heart of cat and rabbit, and controlled his results by determination of the bicarbonate of the perfusate before and after the observation. He states that there was no change worthy of note, but unfortunately gives no figures. Accepting his statement, the results are significant, especially with the hearts of depancreatized cats, where as a result of insulin the quotient rose from a basal average of 0.72 to a mean of 0.90.

For more decisive evidence as to the action of insulin it is necessary to turn to other experiments. In one type chemical analysis is combined with the determination of the respiratory quotient, using the intact animal. This has been admirably done by Bissinger and Lesser (1926) whose analyses included glycogen, intermediary carbohydrates and glucose. They studied the effect of glucose alone as compared with the same amount of sugar plus insulin. The effect of the latter was to decrease the quantity of glucose recovered, and increase the amount oxidized, as judged by the respiratory quotient, to the same degree. Incidentally, glycogen increased at the expense of lower carbohydrate. They conclude that insulin causes glucose to disappear from the normal mouse, and that the quantity which disappears is oxidized. Similar work was done by Cori and Cori (1926, 1927), except that only the glycogen was determined. Chemical analysis and the respiratory quotient together accounted for 90 per cent of the injected glucose. When insulin was given in addition to glucose, less glycogen was formed, and more carbohydrate oxidized.

The fact that in the last two investigations a balance sheet of carbohydrate economy was drawn up, and that the entries tallied closely, is strong evidence of the validity of the conclusions.

Micro-methods are adapted to the study of this problem, because they simplify the experimental conditions. The Barcroft-Warburg manom-

eters obviate the error due to changes in the carbon-dioxide content of the preparation, and permit respiratory and chemical changes to be determined simultaneously. The problem has been attacked by way of the oxygen consumption, or else the respiratory quotient with or without chemical analysis. The former method is inconclusive, unless the increase in oxygen consumption is demonstrated in a medium containing glucose, and not otherwise. This was found to be true of the muscle of the rabbit by Ahlgren (1925) who used an indirect method. This consists in observing the rate at which methylene-blue is reduced in vacuo to a colorless form. Ahlgren's results were confirmed for frog muscle by Blix (1926), using the same method. Brahme (1925) made direct measurements of the oxygen consumption and respiratory quotient of chopped muscle of the frog in pure oxygen, using the micro-spirometer of Thunberg. Before the measurement the muscle was exposed for 5 minutes to a 0.01 per cent solution of glucose in a dilute phosphate mixture at a pH of 7.2 with or without the subsequent addition of insulin. He found an increase in the oxygen consumption which was greatest at concentration (by weight) of insulin "Leo" between 10^{-7} and 10^{-9}, the greatest increase being 44 per cent. Presumably this rise occurred at the expense of carbohydrate, since the respiratory quotient was about 1.0 with or without the extract, implying that the whole of the oxidation was due to carbohydrate in both instances. These results were confirmed by Ahlgren (1926). It is to be noted that the insulin which was used was not a pure chemical. Buchner and Grafe (1924), using the manometric method for determining micro-respiration, obtained evidence that insulin stimulates the oxidation of carbohydrate, but later work from the same laboratory failed to confirm this. Grafe (1925) attributes the earlier results to impurities present in the preparation of insulin. Using similar apparatus in Meyerhof's laboratory Takane (1926) studied the effect of insulin on the diaphragm of the fasting rat. The tissue was immersed in a mixture of horse serum and Ringer solution, or in the former alone. The results as tabulated in Takane's paper are very suggestive, though by no means clear-cut. With insulin both the oxygen consumption and the respiratory quotient rose, suggesting an increase in the oxidation of carbohydrate. This idea was substantiated by chemical analysis. Without insulin, the sugar which disappeared accounted for less than half of the oxygen consumed; with insulin it covered approximately the entire amount.

From the foregoing paragraphs it is evident that insulin causes a rise in the respiratory quotient of intact animals. That this is due to an

increase in the oxidation of carbohydrate is suggested by chemical examination of the entire animal, performed in conjunction with the respiratory exchange. The evidence obtained by micro-respiratory determinations is not clear-cut, but favors the above conclusion, especially if weight be given the work of Takane (1926) in which the determination of the oxygen consumption was combined with chemical analysis.

11. KETOSIS AND THE RESPIRATORY QUOTIENT IN MAN. Ketosis is the formation in the body, or excretion of, the keto-acids, beta-hydroxybutyric and aceto-acetic acid together with smaller amounts of acetone. It occurs whenever too little carbohydrate is oxidized, whether because of diabetes, or as a result of starvation. The mechanism of ketosis has been described in a masterly way by Shaffer (1923) but a brief review may aid in understanding the relation of the respiratory quotient to this subject.

Every medical student is familiar with the old saw, that "fats burn in the fire of the carbohydrates." The problem of theoretical and practical interest is, how much fat will burn in the fire of a given quantity of carbohydrate? When fat fails to oxidize to completion, the products are the ketones, beta-hydroxybutyric acid, aceto-acetic acid, and acetone. Zeller (1914) first obtained quantitative data according to which ketones appeared in the urine of man when 10 per cent or less of the calories of the diet consisted of carbohydrate. Ladd and Palmer (1920), (1923) found the same to occur on the average when 4 grams of fat were present in the diet to every gram of available carbohydrate. These relations suggest a chemical reaction between ketones and glucose, but none was known until Shaffer (1921a) discovered the oxidation in the test tube of aceto-acetic acid in the presence of an alkaline solution of the sugar. Shaffer (1921b) and Woodyatt (1921) worked out formulae for predicting the onset of ketosis. The principles used by the two investigators are the same although expressed by the one in grams and by the other in gram-molecules. Woodyatt aplied his calculations to the diet, whereas Shaffer (1921b) calculated the foodstuffs in the metabolism, using as his data the nitrogen of the urine, the total calories, and the carbohydrate of the diet minus the urinary glucose if present. Both formulae are based on the principle that the foodstuffs may be divided into two categories, one of which gives rise to ketosis, the other prevents it. These are known as ketogenic and anti-ketogenic (ketolytic) respectively. Fat and certain of the amino-acids contained in the protein molecule are ketogenic, whereas all foods which are capable of conversion into

glucose are anti-ketogenic. According to Shaffer (1921c) the assumptions on which the calculations are based are the following:

1. Each molecule of fat is convertible into (a) 3 molecules of aceto-acetic acid and (b) 0.5 molecule of glucose, or its equivalent of antiketogenic derivative.
2. Protein is convertible, (a) into antiketogenic glucose or its equivalent to the extent of 3.6 gm. for each gram of urine nitrogen and (b) into aceto-acetic acid for each molecule of leucine, phenylalanine, and tyrosine, it being calculated that each gram of urine nitrogen corresponds to approximately 10 millimols of ketogenic substance. (c) The amino-acids, valine, lysine, histidine, and tryptophane are neutral as to ketogenesis.
3. Carbohydrate exerts its anti-ketogenic action in the form of glucose or other hexose, 1 gm. of which is therefore equivalent to (1,000 ÷ 180 =) 5.56 millimols of antiketogenic substance.

Under 2 (a) in the preceding paragraph the figure 3.6 is the dextrose nitrogen ratio observed in diabetes of maximum severity. Under 2 (b) the quantity of keto-acid derivable from protein is based on Osborne's and Jones (1909) analysis of ox muscle, as modified by Lusk (1915b). Shaffer (1921b) summarizes the calculations as follows:

Ketogenic substance expressed as gram molecule of precursors of acetoacetic acid.

(a) 1 gm. of fat $= \frac{3 \times 1}{874} = 0.00343$ mol.

(b) 1 gm. of urine nitrogen = 0.010 mol.

Antiketogenic substance expressed as gram-molecular equivalents of glucose.

(c) 1 gm. of urine-nitrogen $= \frac{3.6}{180} = 0.020$ mol.

(d) 1 gm. of glucose from carbohydrate $\frac{1}{180} = 0.00556$ mol.

(e) 1 gm. of fat $\frac{1}{874} \div 2 = 0.00057$ mol.

Of these, (a), (d) and (e) are simple calculations involving only the molecular weight. (b) is from the modified Osborne table above mentioned. (c) is from the D:N ratio.

A similar formula expressed in grams was published by Woodyatt (1921). It was inverted in the original paper, but should read:

$$\frac{\text{fatty acid}}{\text{glucose}} = \frac{0.46 \times \text{protein} + 0.9 \times \text{fat}}{\text{carbohydrate} + 0.58 \times \text{protein} + 0.1 \times \text{fat}}.$$

This formula works equally well. The ratio obtained is not molecular but may be made so by dividing by 1.5. Some confusion may arise from

the fact that the ketogenic substance is expressed as fatty-acid, although the amino-acids obviously do not pass through this stage. The use of the term is to be regarded as a chemical fiction, for which there is ample precedent. Woodyatt found, in agreement with Shaffer (1921b) that ketosis could be avoided so long as one molecule of glucose was oxidized for every molecule of keto-acid. (The following citations are also in terms of molecular ratios.) McCann, Hannon, Perlzweig and Tompkins (1923) found a minimum ratio of 1.0, but frequently higher. Wilder and Winter (1922) and Ladd and Palmer (1920, 1923) observed ketosis only when the molecular ratio exceeded unity. Wilder, Boothby and Beeler (1922) found with their patient a ratio of 1.5 when the excretion of the acetone bodies was 2.4 grams per 24 hours. Weeks, Renner, Allen and Wishart (1923) gave immense quantities of fat to epileptic patients without serious ketosis as judged by qualitative analysis of the urine, together with the alkali reserve of the blood. Using the same criteria for ketosis, Mason (1927) studied obese individuals during semistarvation, and applied Shaffer's formula (1921b) to the estimated total calories, the nitrogen excretion, and the carbohydrate intake. With each of 5 individuals he obtained ketogenic-anti-ketogenic ratios (molecular) reaching 2.0 or over, without causing ketosis. Respiratory quotients were not reported, but would undoubtedly have been in the neighborhood of 0.70.

The above observations were partially dependent on the diet. Shaffer (1921c, table III) extended his calculations to respiratory data, and constructed a table by which the ketogenic-anti-ketogenic ratio can be read off with sufficient accuracy from the gross respiratory quotient. The smaller the latter, the greater the quantity of fat in the metabolism, and the higher the ketogenic ratio. Shaffer (1921c) concluded that "a molecular ratio of 1:1, which corresponds (according to the method of calculation) to a respiratory quotient of 0.76, is the limit for the avoidance of the excretion of the acetone bodies." Inspection of the table compiled by Joslin (1923a, table 82) reveals that this same quotient of 0.76 is in the border zone of ketosis. Richardson and Ladd (1924) applied the principles above described to data obtained with the respiration calorimeter, with the same results.[2] Similar observations were made by Richardson and Levine (1925a) on the effect of infections. It was found that the latter produced no derangement of the mechanism of the ketogenic balance either in diabetic or non-diabetic individuals, although Wilder and Winter (1922) using calculations based in part on the respiratory exchange, had concluded that infection depresses the threshold of ketogenesis.

[2] See also McClellan, Spencer, Falk and Du Bois, p. 125, addenda.

So far the discussion has been limited to the ratio necessary to prevent the onset of ketosis. In attempting to account quantitatively for the ketones excreted, Shaffer (1922) found less than was to be expected from the excess of ketogenic over antiketogenic molecules. He altered the formula by increasing the ketogenic value of protein 50 per cent and multiplying the total mol. of anti-ketogenic substance by 2 and obtained better agreement between the theoretical and actual results. It would be interesting to check these results by means of the respiratory quotient, but unfortunately the latter is unreliable in the presence of severe ketosis.

Evidently it is essential to distinguish between the ratio necessary to prevent the excretion of the acetone bodies, and that which is required to account for the quantities of ketones excreted. With regard to the former, it is to be pointed out that most of the observations based solely on respiratory data are in agreement that the threshold ratio is 1:1, although exceptions do occur as in some of the observations of Joslin (table 82, 1923a). Richardson and Ladd (1924) found that the ratio computed from the diet may be twice as high as that calculated from a simultaneous rspiratory quotient, and this may account for some of the higher ratios noted at the threshold of ketosis on the basis of incomplete respiratory data. Shaffer (1922) has another explanation. According to this the actual ratio is one molecule of glucose (or two of triose) to each two molecules of ketone, and the existence of lower ratios at the threshold is due to lack of a proper mixing in the body.

Apparently this subject has not received the same amount of attention in Europe, although Geelmuyden (1923) expressed much the same idea, as early as 1904. He considered that ketones and the carbohydrates unite by a synthesis which is necessary for the further oxidation of the former. Later he modified this hypothesis with the suggestion that the ketones do not burn direct, but are first synthesized to sugar and glycogen. If, however, fats were converted into carbohydrate, through the ketones or otherwise, it is not clear why any threshold ratio should exist, nor why carbohydrate should be necessary for the oxidation.

Petrén (1925) with the help of Odin has studied the question by determining the carbon-dioxide of the blood and the acidity of the urine of diabetic patients. He concludes that there are two kinds of acidosis, one common to all individuals undergoing carbohydrate starvation, the other confined to diabetes. The latter is serious and is caused by the excess of protein in the diet. Fat, according to this author, may have no ketogenic effect, and butter fat is actually beneficial.

It is a pity that a theory as novel as this was not supported by a greater variety of experimental data, in particular the respiratory quotient. It is quite possible that the fat was stored rather than oxidized, and lacked ketogenic effect for this reason alone. Petrén's distrust of protein is shared by a number of authors, for instance Gephart, Aub, Du Bois and Lusk (1917) and Wilder, Boothby and Beeler (1922) but may be justified on the theory of ketogenesis. In the first place, in very severe diabetes the ketogenic factor of protein becomes predominant, because the glucose derived from this food fails to become oxidized. Secondly, ingestion of large quantities of protein stimulates the total metabolism and with it the oxidation of fat.

In summary, the fundamental equation by which the formation of ketones is prevented is unknown. Apart from the in-vitro analogy brought forward by Shaffer (1921a) and recently extended by West (1927), the assumptions on which the theory of the ketogenic balance is based have received no further verification. In spite of these limitations, however, it is evident that in man some quantitative relation exists between the oxidation of fat and that of carbohydrate, and this is known with sufficient accuracy to make the theory of the ketogenic balance all but indispensable in the treatment of diabetes.

12. Renal glycosuria. Renal glycosuria or renal diabetes is a disease characterized by a low blood-sugar in the presence of large quantities of glucose in the urine. In contradistinction to diabetes, the glycosuria is independent of the diet. Joslin (1923b) suspects that many patients supposed to have renal glycosuria subsequently develope diabetes. Any method of differentiating the two conditions is therefore of great practical interest. The blood-sugar curve following the ingestion of glucose has been widely used for this purpose, although it has no necessary relation to the quantity of carbohydrate oxidized. This objection does not apply to the curve of the respiratory quotient obtained under the same conditions. In the normal individual, as mentioned above, the ingestion of glucose is followed by a prompt and prolonged rise in the respiratory quotient, showing an increase in the combustion of carbohydrate. In severe diabetes no such effect is noted. The nature of renal glycosuria may therefore be judged, according as the reaction of the patient to the ingestion of glucose follows the normal or the diabetic type. Finley and Rabinowitch (1923–24) noted the effect of the ingestion of glucose on the respiratory quotient, and found a sharp rise to a maximum of 0.92, indicating a marked increase in the oxidation of carbohydrate. The patient studied by Ladd and Richardson (1925)

sustained a rise in the quotient to the same level. The non-protein respiratory quotient was 0.96, showing the oxidation of carbohydrate to the almost complete exclusion of fat. Rabinowitch (1925) observed a marked increase in the quotient. In four of the five patients the rise was between 0.10 to 0.17. In only one of the eleven diabetics studied by the same method was there an increase of the same order. In renal glycosuria, therefore, the patient reacts to the ingestion of carbohydrate in the manner of the normal individual rather than the diabetic. There appears to be no ground for believing that renal glycosuria is a preliminary stage of diabetes mellitus.

CONCLUSION

The scope of the respiratory quotient is broad, including as it does the whole process of the utilization of food, excepting only digestion and absorption. This may be illustrated by the following reaction:

Foodstuff plus $O_2 \rightarrow$ intermediary reactions a, b, c, ...n $\rightarrow CO_2 + H_2O +$ unoxidized residue.

The respiratory quotient is the relation in volumes between the CO_2 given off and the oxygen consumed, and obviously gives no information as to the intermediary steps in the oxidation. Moreover, the equation fails to take into account an important factor, the net change in gaseous content of the body.

Table 3 is a diagram of the influences which play upon the respiratory quotient. At the left the CO_2 and O_2 measured refer to the data obtained in a reliable metabolism experiment, in which technical accuracy may be assumed. Part of the CO_2 which is measured is derived from oxidation, but another portion may be due to expulsion from the body, either by increased respiration, or by accumulation of acid. To obtain the true or metabolic CO_2 it is necessary to subtract from the total the quantity expelled. Conversely, when acid disappears from the body in the period of an experiment, CO_2 is retained, and this amount must be added to the figure obtained by measurement. Unfortunately, reliable data for making these corrections cannot be obtained. Various influences which affect the quantity of acid in the body are detailed in the diagram. It may seem confusing that oxidation of acid appears in the portion of the chart devoted to non-oxidative influences, but it is the removal of the acid which is considered here, not the means.

When the influence of acid has been allowed for, or found not to exist, there remain the CO_2 and O_2 due to oxidation, and the former divided by the latter gives the true or metabolic quotient. This is influenced

TABLE 3

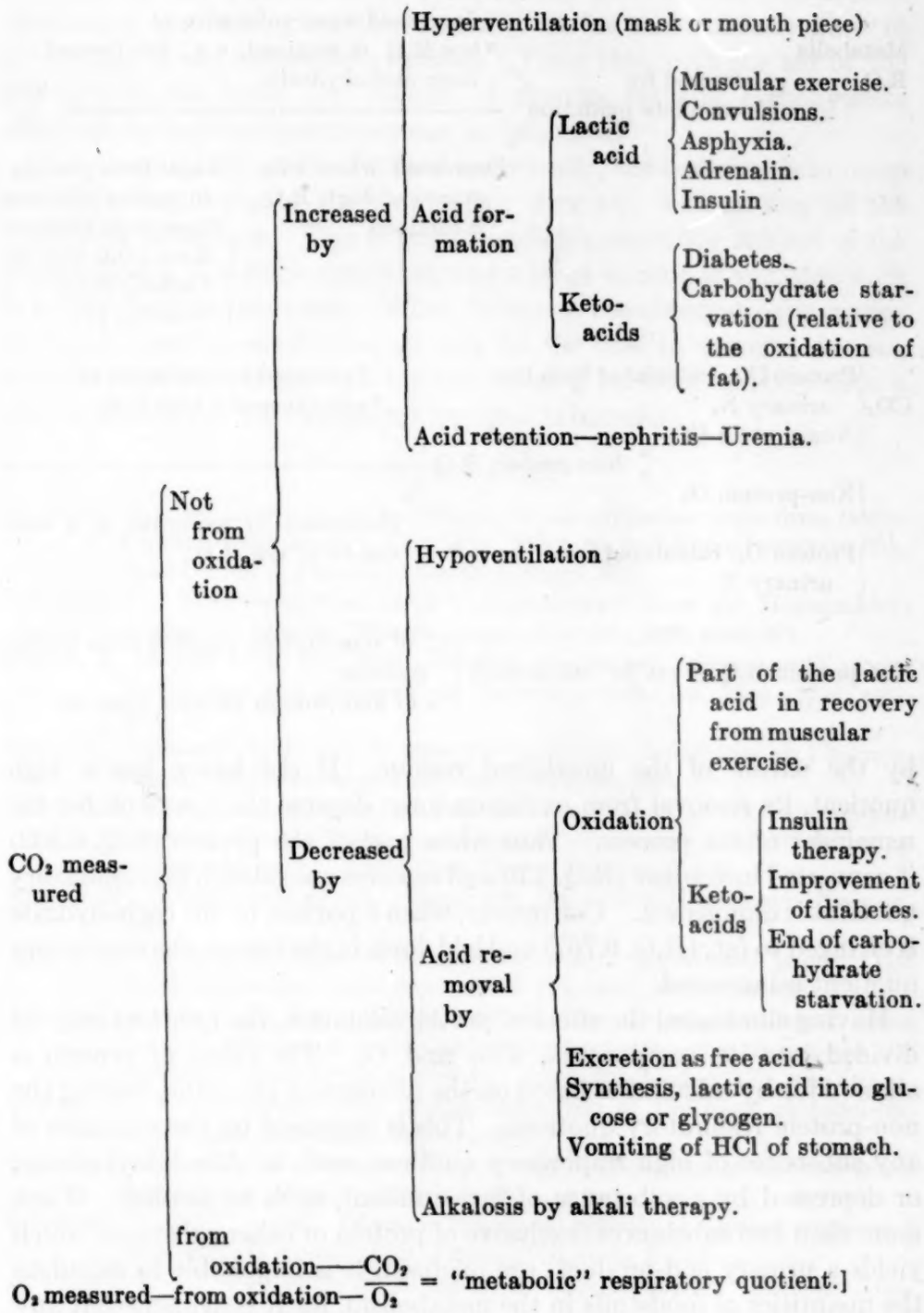
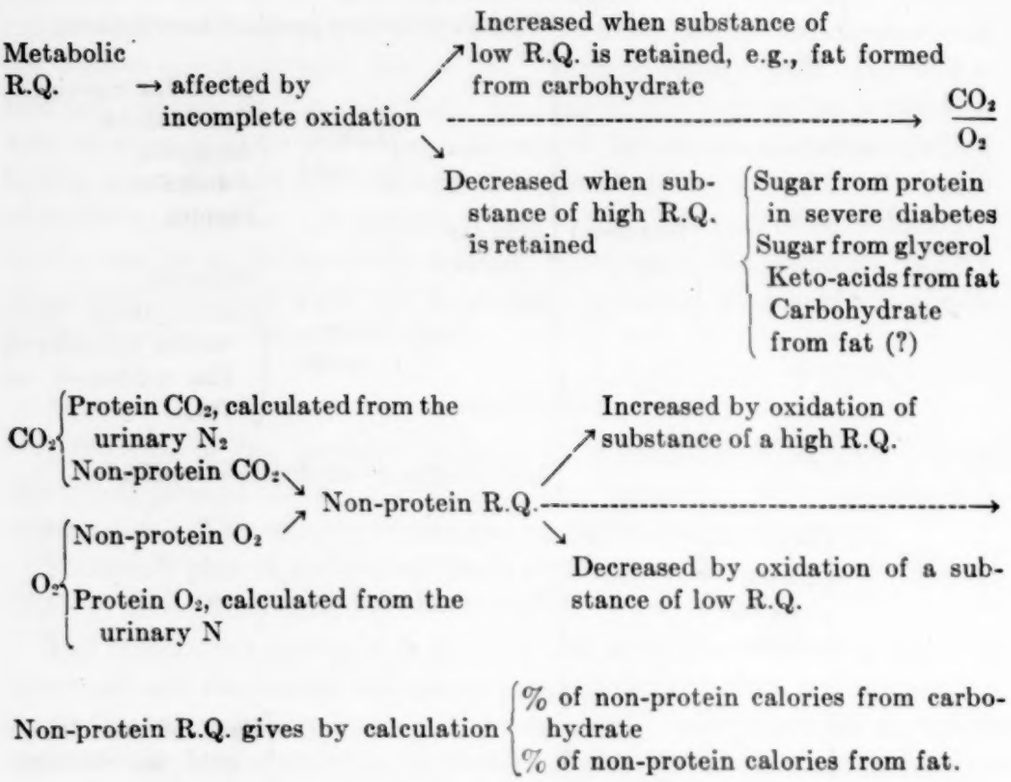

Hyperventilation (mask or mouth piece)
Increased by
Acid formation
Lactic acid
Muscular exercise.
Convulsions.
Asphyxia.
Adrenalin.
Insulin
Keto-acids
Diabetes.
Carbohydrate starvation (relative to the oxidation of fat).
Acid retention—nephritis—Uremia.
Not from oxidation
Hypoventilation
Part of the lactic acid in recovery from muscular exercise.
Decreased by
Oxidation
Keto-acids
Insulin therapy.
Improvement of diabetes.
End of carbohydrate starvation.
Acid removal by
Excretion as free acid.
Synthesis, lactic acid into glucose or glycogen.
Vomiting of HCl of stomach.
Alkalosis by alkali therapy.
CO2 measured
from oxidation
O2 measured—from oxidation—CO2/O2 = "metabolic" respiratory quotient.]

TABLE 3—*Concluded*


Metabolic R.Q.
affected by incomplete oxidation
Increased when substance of low R.Q. is retained, e.g., fat formed from carbohydrate
CO2/O2
Decreased when substance of high R.Q. is retained
Sugar from protein in severe diabetes
Sugar from glycerol
Keto-acids from fat
Carbohydrate from fat (?)
CO2
Protein CO2, calculated from the urinary N2
Non-protein CO2
O2
Non-protein O2
Protein O2, calculated from the urinary N
Non-protein R.Q.
Increased by oxidation of substance of a high R.Q.
Decreased by oxidation of a substance of low R.Q.
Non-protein R.Q. gives by calculation
% of non-protein calories from carbohydrate
% of non-protein calories from fat.

by the nature of the unoxidized residue. If the latter has a high quotient, its removal from oxidation must depress the quotient for the remainder of the process. Thus when part of the protein (R.Q. 0.802) is converted into sugar (R.Q. 1.0) and remains unoxidized, the respiratory quotient is diminished. Conversely, when a portion of the carbohydrate is changed to fat, (R.Q. 0.707) and laid down in the tissues, the respiratory quotient is increased.

Having eliminated the effect of partial oxidation, the quotient may be divided into its components, CO_2 and O_2. The effect of protein is allowed for by calculation based on the nitrogen of the urine, leaving the non-protein respiratory quotient. This is increased by the *oxidation* of any substance of high respiratory quotient, such as dihydroxyacetone, or depressed by a substance of low quotient, such as alcohol. When more than two substances (exclusive of protein or other substance which yields a urinary end-product) are oxidized, it is impossible to calculate the quantities of foodstuffs in the metabolism, for it is evident that any given respiratory quotient may be the result of varying combinations. In spite of this, inferences may be drawn in a qualitative sense.

When no third substance is present, the level of the non-protein respiratory quotient gives the quantity of fat and carbohydrate in percentage of the non-protein calories. From this, with the aid of the nitrogen of the urine, the calories of protein, fat and carbohydrate oxidized during the observation can be calculated.

If the respiratory quotient is used uncritically, the tendency is to jump from the beginning to the end of the diagram, disregarding all the modifying influences. This is quite justifiable when the subject of the observation is in a static condition, and a large volume of valuable work has been done on this basis. When, however, the observation is carried on under varying conditions, as may be the case in exercise, ketosis, diabetes, insulin therapy, and the formation of fat from carbohydrate, a critical scrutiny of the respiratory quotient is in order.

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ADDENDA

MCCLELLAN, W. S., H. J. SPENCER ET AL. Clinical Calorimetry. XLIII. A comparison of the thresholds of ketosis in diabetes, epilepsy and obesity. J. Biol. Chem. (In press for Dec. 1928.)

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BIOCHEMICAL INVESTIGATION OF VITAMIN B

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More attempts have been directed toward the isolation of vitamin B than toward any other member of the interesting but elusive series of dietary factors, the vitamins. When it is remembered that the existence of this principle has been recognized for more than three decades, there is ready reason for the enormous amount of effort expended toward elucidating its chemical nature. Further, its remarkable stability, particularly to heat and oxidation, in contrast to most of the other vitamins places it in a favored position for investigation, unless the recently discovered vitamin E be considered. Even then, vitamin B possesses a decided advantage in that it has the more desirable medium, water, for chemical manipulation. All these circumstances have contrived to make this vitamin the principal point of chemical attack, and there have been few biochemists in recent years who have not been drawn into this field, if only for a brief excursion.

Unlike its kindred nutritional factors, vitamin B commanded considerable attention long before the vitamin hypothesis sprang into being. It may seem anomalous that the first known principle should receive second place in the nomenclature. However, vitamin B was first recognized as a disease preventive and only later as a dietary essential for growth. The correlation of apparently conflicting but actually concordant data leading to this final conclusion involved a long delay during which vitamin A was discovered. It is interesting that one man exerted a tremendous influence upon the discovery of the antineuritic substance and later foreshadowed the vitamin hypothesis, although both in an indirect way. In 1887 after a Netherlands commission had completed its investigation of an outbreak of beriberi in the East Indies colonies, Professor C. Pekelharing induced the government to establish a laboratory in Weltevreden, Java, for the study of such disorders (143). There two years later Eijkman, the first director, observed a disease in fowls which was analogous to beriberi.

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In 1905, long before the existence of unknown, indispensable items in foodstuffs was commonly recognized, to say nothing of any possible relationship between Eijkman's neuritic phenomenon and these dietary principles, Pekelharing was among the first to hazard the opinion that " . . . . there is a still unknown substance in milk, which, even in very small quantities, is of paramount importance to nourishment" (251). By these apparently unassociated contributions Pekelharing looms large in the background of nutrition, and by a strange turn the passage of time has placed the antineuritic substance, which cures Eijkman's experimental beriberi, in the category with the dietary essentials.

The disease resembling beriberi in its paralytic symptoms and manifesting much the same pathological degeneration of nerves was seen by Eijkman in fowls which had been fed scraps from hospital fare. After investigation into its etiology, he was able to produce the disorder at will by a diet of polished rice, in which the grain is devoid of pericarp and embryo (68). Later he showed that starch-rich foods likewise led to this disease, which he called *polyneuritis gallinarum* (70). Cure and prevention were effected by partially polished or natural rice. Since in partially milled rice only the coarse outer coating is removed and the inner "silverskin" is left intact, he reached the conclusion that the virtue of this product lay in the skin, and that polished rice produced polyneuritis because it lacked this essential layer (69). Concerning the mechanism, Eijkman postulated that starch in the cereal grain gave rise to toxins which exerted a deleterious effect upon the nervous system. In the silverskin of the kernel was contained a substance or substances which would neutralize these toxins. His intoxication theory was soon challenged, and in the light of the present knowledge of nutrition it is evident that his interpretation was incorrect. His observations, however, were sound and they established him as the first to produce experimentally in animals a disease of dietary origin.

This work was soon repeated and extended by Grïjns (139). He induced polyneuritis in fowls by feeding them meat which had been heated for several days at 100°, or for a short time at 120°C. Further, he found that a small quantity of the native bean, katjang idjo, was an excellent preventive of the disease. Following this demonstration that ingestion of heated meat, something other than starches, could cause polyneuritis, Grïjns rejected Eijkman's amylum-toxin theory and attributed the disease simply to a dietary deficiency. It was Grïjns' belief that certain unknown substances were necessary for the metabolism of nerves and that they were distributed unevenly among

foodstuffs, much in katjang idjo but very little in polished rice. When food was deficient in this respect, polyneuritis made its appearance. In recognizing the existence of an indispensable antineuritic principle, which was entirely different from known substances deemed essential to normal nutrition, Grĳns established the view that is substantially held at the present time.

Unable to successfully repeat Grĳns, production of polyneuritis by sterilized meat, Eijkman maintained his stand that the presence of starch was necessary for the origin of the disease (71). Continuing upon this assumption, he attempted to isolate the counteractant from rice bran. He found that an aqueous extract of the bran possessed therapeutic properties, but that phytin, a phosphorized product contained in it, was without effect. The curative principle, he learned, was dialyzable and not precipitated by alcohol. At this time Grĳns felt constrained to point again to the improbability of the toxin theory upon the grounds that so many fundamentally different substances, meat, rice, and starches, would scarcely form the same toxin, and that the antidote would not likely be present in so many foodstuffs (140, 141).

The toxin hypothesis was subjected to a direct test when Fraser and Stanton attempted to extract poisonous substances from white rice which had actually been associated with outbreaks of beriberi (93). In this purpose they failed, but they did not disturb thereby the validity of Eijkman's theory. Their histological examination of rice grains showed that remnants of the pericarp remained attached to the parboiled kernel, but that in white rice the pericarpal and subjacent layers had been polished away. When the parboiled rice, which was preventive for polyneuritis, was exhausted with hot alcohol, it lost its efficacy and prompted the appearance of the disease. White rice, which alone was likewise ineffective, prevented polyneuritis when supplemented with the alcoholic extract from parboiled rice. Unfortunately, these experiments supported the theories of Eijkman and Grĳns equally well.

In some notes upon the etiology of beriberi, Durham dropped a random suggestion about the possible importance of "a sufficient amount of assimilable phosphorus in the diet" as a defense against the neuritic toxin (52). A subnormal excretion of phosphorus compounds by patients suffering from beriberi led Schaumann to attach significance to the intimation. Soon he discovered that katjang idjo, a prophylactic for the disease, increased the amount of phosphoric acid in the urine (270). This finding strengthened his conviction concerning the value of phosphorus, since he ascribed the beneficial results from the native

beans to their relatively high content of phosphoric acid. From a theoretical consideration of changes which food might undergo upon storage, heating, or extraction, and which might leave it unable to cover the phosphorus needs of the body, Schaumann framed his hypothesis that beriberi was a dietary disease arising from a deficient supply of organically bound phosphoric acid, probably nucleic acid.

Further investigation showed that pigeons failing on polished rice were not protected by phosphates, metaphosphates, or yeast nucleic acid, although the latter exerted temporary curative effect (271). Yeast and wheat bran, which are rich in nucleins, prevented the occurrence of the neuritic disorder. Confronted with these results, Schaumann was led to enlarge his view slightly concerning the specific phosphorus compounds at work. He stated that nucleo-proteins, perhaps with the assistance of phosphatids, were the salutary factors in yeast and wheat bran. But Grijns isolated the nucleins from katjang idjo beans, and found these substances to be inert towards polyneuritis. The filtrate, supposedly nuclein-free, still possessed some activity, although considerably reduced (142).

Schaumann was not alone in his belief that a causal nexus existed between organic phosphorus deficiency and the incidence of beriberi. After finding that addition of phytin to polished rice prolonged the life of chicks, Aron believed that one of the main factors in protective substances must be an organic compound of phosphorus (10). Although not controverting Schaumann that nucleo-protein might be the active substance in yeast, Aron was inclined to the view that in rice it was the phosphorus compound, phytin. Neither Schaumann (272), Fraser and Stanton (94), nor Eijkman (72) could corroborate this success with phytin. That much uncertainty existed at this time concerning the nature of the active organic compounds of phosphorus is quite apparent. Schaumann became somewhat less definite in his designation of the essential substances (272). Calling attention to his unsatisfactory results with such compounds as yeast nucleic acid, phytin, and certain phosphatids, which had been separated from natural protective substances (yeast, katjang idjo, and rice bran), he stated that apparently the protective or curative effect of the foodstuffs depended upon the collective action of a number of organic compounds of phosphorus, rather than upon any one. The identity of these substances was mentioned as uncertain.

With the phosphorus theory occupying the foreground of beriberi research, Fraser and Stanton determined the phosphorus content of

various kinds of rice, and drew the correlation that the higher the amount the less likelihood there was for the grain to produce polyneuritis (93). Their conclusion that white rice lacked some substance or substances essential for the metabolism of nervous tissue was in line with Grĳns' views. They gave no intimation that these stubstances might be phosphorus compounds; indeed they were careful to mention that a phosphorus determination served merely as an indicator. This precaution was well taken, since their later systematic examination of extractives from rice polishings showed that phosphorus compounds equal to 85 per cent of the total phosphorus content were almost without effect in preventing polyneuritis (94), a fact which was attested by Chamberlain and Vedder (24). Although these results dealt a severe blow to Schaumann's hypothesis, the possibility was not excluded that the remaining 15 per cent phosphorus was in a particular combination which made it essential. This view was soon made unlikely by Chamberlain and Vedder's preparation of an active extract from rice polishings which contained only 0.00165 per cent phosphorus pentoxide (24). There lay evidence that at least 999 parts of each 1000 parts of phosphorus were unimportant in the prevention of polyneuritis in fowls. Their conclusion that it was "impossible to state positively that this minute trace of phosphorus remaining in the extract is not a neuritis-preventing element but . . . . that it appears incredible that it can be of slightest importance" certainly shows the utmost restraint upon their part. Whether further effort in this direction was justifiable is best judged by Eijkman's pointed comment that if Schaumann had borne in mind the insolubility of nucleins, nucleo-proteins, and nucleic acid in strong alcohol, a solvent for the antineuritic substance, he would have found it difficult to formulate his nuclein-phosphoric acid hypothesis (72).

In the face of active preparations low in phosphorus (24, 328) Schaumann was forced again to modify his conception. If such small amounts of phosphorus in the preparations possessed any value for beriberi, it was certainly not that they were "Bausteine" for the formation of body materials (273). Rather he now viewed the antineuritic principle as an enzyme, cleaving phosphorized foodstuffs; or as a catalyzator, promoting resynthesis of the products. In general, the protection against beriberi was seen as a combined effect involving the presence of his new factor, the activator, and organic phosphorus compounds; the former was responsible for complete utilization of the latter (275).

As the result of a chemical investigation instituted in connection

with his new notion, Schaumann separated two distinct phosphorized products, in each of which the protective principle was regarded as the same; later he identified the activator in the preparations as a nitrogenous substance, free from phosphorus (274). It is a long way from Schaumann's original assertion that the protective substances were organic compounds of phosphorus, used as such, to his statement at this time that "whether activator itself contains phosphorus is not important, its real value is its indirect effect upon metabolism by facilitating the assimilation of certain foodstuffs."

The true relation of the antineuritic principle to the occurrence of beriberi was more fully grasped after Funk had reported the separation of an extremely potent antineuritic preparation which was entirely phosphorus free (104). His findings did not shed any light upon the validity of Schaumann's new interpretation, which was somewhat impervious to cogent proof, but it did substantiate Grĳns' theory that beriberi and polyneuritis were due to starvation for a specific substance. Funk demonstrated in a spectacular manner that only a surprisingly small amount of the substance was necessary to prevent the appearance of the nervous symptoms characteristic of the disease (104). It was beginning to be appreciated more fully just then that certain essentials must be included in the diet in addition to the long recognized constituents, *viz.*, protein, carbohydrates, fats and mineral elements. This trend was reflected rather sweepingly in a statement by Funk that several deficiency diseases could be prevented and cured by addition of specific compounds, organic and basic in nature, which he called "vitamines" (105). In the list of such disorders he placed beriberi, sprue, rickets, and pellagra. In its revamped form the deficiency theory thus became the "vitamine" hypothesis; and beriberi became a disease, among others, due to the lack of a "vitamine" distributed in certain food stuffs. Funk's concentrate contained this antineuritic "vitamine."

The almost dramatic cures effected by infinitesimal amounts of the "vitamine," together with the magic of its name, stirred popular imagination and stimulated research anew on the beriberi problem; but Eijkman was not yet ready to accept the vitamin interpretation. He was not willing to admit that the toxin theory had been disproved, although practically all of the other workers now regarded that matter as settled (73). He did, however, at this time disclaim sponsorship for the toxin theory, stating that his purpose was merely to raise this as a possibility. It was apparent that his stand was weakening, but it was not until 1916 that he abandoned the intoxication theory from lack of evidence of endogenous toxin formation (74).

It must not be supposed, however, that Eijkman's conversion to the vitamin hypothesis brought unanimity on this score. Despite the number of instances in which polyneuritis had been cured by extremely small amounts of unknown substances "isolated" from foodstuffs, Abderhalden and his collaborators were long of the opinion that there was insufficient evidence for the assumption of such indispensable principles. They viewed polyneuritis as an intoxication resulting from intestinal obstruction (1). By 1920, however, Abderhalden agreed that there was no longer doubt of the existence of a series of nutritive elements which participate in metabolism and influence organic functions in quite a specific way (4). This position was reaffirmed when pigeons contracted polyneuritis from a purified diet as readily as they did from polished rice. The purified ration would scarcely have a poisonous effect; it was more likely to lack indispensable substances (5).

Abderhalden's belated recognition of the vitamin theory left von Hahn alone unwilling to subscribe to its tenets. This latter investigator denies the specificity of individual vitamins for such diseases as beriberi and scurvy (145). In fact, he believes that no chemically definite substance functions as a vitamin, but that a class of substances is able to exert a curative effect by its common property of surface activity. By this mechanism, he states, the active substances permit passage of foodstuffs in a dispersed condition through the membrane of the intestinal wall, *i.e.*, absorption. In avitaminosis there is a decrease in surface activity which leaves the membrane impermeable to the colloidal particles, but the small amount of molecularly dispersed foodstuffs which is absorbed determines the nature of the deficiency disease. Substances which promote permeability of the membrane are in a vitaminoid condition. Curiously a comparison of the surface activity and vitamin content of foods showed an almost complete parallelism (146). Further, surface activity varied directly with vitamin potency after heat treatment and in the various steps of isolation (147). Biochemists engaged in the study of the vitamins, however, have shown no inclination to adopt this view.

NOMENCLATURE. That an account of the chemical investigation of vitamin B may be intelligible, it is necessary to gain an insight into its complex and all too extensive terminology. Prior to 1911 none of the earlier workers attempted to name the essential substance or substances. In that year Schaumann designated his active preparation "activator," but the term did not come into widespread use (275). At the same time Funk separated a potent product from rice polishings (104), and postu-

lated the existence in foodstuffs of a number of such indispensable substances which he called "vitamines" (105). Contemporaneous with Funk's work appeared a publication by Suzuki, Shimamura and Odake on the isolation of an antineuritic substance "oryzanin" (321); and another by Edie, Evans, Moore, Simpson and Webster on a similar product which they named "torulin" (57). Further, Tsuzuki described a patented preparation as "antiberiberin" (331).

All these names had been given to the specific substance in foods which protected against the particular disease, beriberi. In 1912 Hopkins called attention to the necessity of "accessory factors" in the diet for growth (165). Within a short time McCollum and Davis had demonstrated that certain fats contained such a substance required by rats for development and well-being (217a). This Funk called the "growth vitamine," and like other investigators at that time he was under the misapprehension that it was the only unknown substance necessary for growth in the rat (114). Later McCollum and Davis showed that these animals when fed a supposedly adequate diet containing a source of the fat factor failed in growth (217). An investigation led them to conclude that there was a second essential for normal nutrition during growth and that it was distinct from the substance present in fats. This new factor was found in rice polishings and wheat embryo; it was soluble in water. They expressed the view that there were two classes of unidentified substances needed by the rat, aside from protein, carbohydrate, fat and salts; the one was soluble in fats, the other in water (218). Continuing this approach, McCollum and Kennedy adduced evidence to show that polyneuritis in pigeons and failure of growth in rats on a supposedly complete diet were due to lack of one and the same substance, the factor soluble in water (220). This identified Funk's antineuritic "vitamine" with McCollum's water-soluble dietary essential.

In an attempt to secure a satisfactory system of nomenclature for the dietary essentials which would be based upon the nutritional requirements of the rat, McCollum and Kennedy advised the discontinuance of the term "vitamine." Their criticism was leveled against the prefix *vita* because it connoted a physiological importance for these substances paramount to that of certain other equally indispensable complexes; and against the suffix *amine* because it conveyed the unwarranted implication of an amine nature for the dietary principles. Instead they proposed the use of the terms "fat-soluble A" and "water-soluble B" for the two classes of substances concerned in inducing growth. The merits claimed for this system were: (a), no implication of

extravagant values for these nutritional factors; (b), differentiation upon the only known basis, solubility; (c), provision for expansion. Drummond suggested a modification of this scheme, which has been generally adopted (50). In it the final "e" was dropped from vitamine, making the acceptable word vitamin. This designation was linked to McCollum's system of letters to denote the groups. Water-soluble B thus became vitamin B, the name given to both the antineuritic substance and the growth-promoting principle.

Funk has preferred to retain the original word "vitamine." He has accepted, nevertheless, the alphabetical termination, except for a few occasions when he has referred to "vitamine B" as the "antineuritic" or "antiberiberi vitamine" (124). Upon the assumption that the antineuritic constituent and the growth factor were distinct and separate entities, Abderhalden and Schaumann chose the name "eutonine" for the substance specific for nervous disturbances, and assigned the collective term "nutramine" to all the unknown but indispensable constituents which limit the biological value of a diet (3). Hofmeister referred to the active principle as "antineuritin" but he gave the designation "oridin" to an isolated product, the physiological activity of which disappeared upon purification (161). For the antineuritic substance Peters used the name "torulin", originated by Edie and collaborators, but he limited its application to the product that cures head retraction in pigeons (254).

In 1919 R. J. Williams advanced the claim that Wildier's "bios," a substance stimulating growth in yeast, was identical with vitamin B (349). MacDonald and McCollum (210), and Fulmer, Nelson and Sherwood (97, 99), on the contrary, became convinced that these two principles were not the same. They admitted that there was a bios, but they asserted that its absence was immaterial to the yeast organism. It could not, therefore, function as a vitamin, which is absolutely essential for growth or well-being (99, 213). Funk and Dubin, however, claimed that bios was indispensable for yeast growth, and that accordingly it functioned as a "vitamine" (123). They introduced the term "vitamine D" for it, although this designation has been generally applied to McCollum's antirachitic factor (224a). Somewhat different was von Euler's distinction between the vitamins, by which he meant the substances protecting against avitaminoses, and growth factors which he called "biocatalysators" (82). For the vitamins he employed the usual letters, but he divided Funk's vitamin D into two groups. One comparable to bios was called h D_m; the other characterized by its

growth effect upon rats was designated h D_r (83, 84). Then we must mention Burrows and Jorstad's "archusia", the non-specific substance necessary not only for growth but also for all activities of a cell (23a). To these workers "vitamin B is probably only the normal energy for life formed by all cells in nature."

At the same time that the bios complication was under investigation with regard to its relation to vitamin B, Mitchell drew a distinction between the antineuritic vitamin curing polyneuritis in pigeons and the water-soluble B vitamin promoting growth in rats (233). Although this paper presented only an interpretation of previously published data drawn from many sources, evidence of a satisfactory nature was soon forthcoming and the notion of the multiple nature of vitamin B gained much ground. When Goldberger and his co-workers first postulated the existence of a substance, P-P, preventing pellagra, it seemed to have slight connection with vitamin B (135). Later they advanced the belief that water-soluble B, *i.e.*, the growth-promoting substance, consisted of two factors; the one an antineuritic principle for which they retained the term vitamin B in *sensu strictu*, and the other a pellagra preventive which they had already named P-P (136). The best recognized chemical distinction between them was their differential behavior to heat; the vitamin B was thermolabile, the factor P-P was thermostable.

In elaborating upon Goldberger's theory, Salmon has suggested that the thermolabile antineuritic substance be given the designation B-P, beriberi preventive, instead of vitamin B; that the thermostable substance keep the term P-P; and that the name vitamin B be assigned to the complex of the factors (268). Another variation was introduced when Chick and Roscoe designated the complex as water-soluble B vitamin, comprising the thermolabile antineuritic factor which they termed antineuritic vitamin, and the thermostable antipellagrous factor which they called vitamin B *sensu strictu* (30). Plimmer and his colleagues presented objections to this nomenclature and urged the return to Goldberger's system (256). The existence of two factors was recognized by Sherman and Axtmayer who treated them as two primary vitamins rather than as subordinate parts of a vitamin complex (303). Accordingly, they suggested vitamin F (rather than B or B-P) for the antineuritic factor, and vitamin G (rather than B or P-P) for Goldberger's thermostable pellagra-preventive. But this implied the use of vitamin B as a group name for the F and G substances. Smith has recently expressed her preference for this system (307a).

Little mention is needed that vitamin B nomenclature at the present time is almost hopelessly confused. Upon the assumption that vitamin B is a complex of at least two factors, the term "vitamin B" has been applied to each of the factors respectively by two different groups of investigtors and to the complex by still another. Thus, vitamin B is the antineuritic factor for Goldberger; it is the pellagra-preventive and growth-promoting factor for Chick and Roscoe; it is the combination of the two for Salmon. It is not uncommon for each investigator to preface his paper with his system of nomenclature; then in order to render it more intelligible to express its equivalent in terms used by other workers. Added to the fact that investigators have given different names to the same substance, often they are not certain that they are dealing with the same substance, due to difference in test method. It presents a situation that sorely needs clarification.

Funk has evolved a classification which he suggests will contribute to this end. Under this system the dietary essentials would be separated into two groups: one containing nitrogen and unstable to alkali treatment would retain his designation "vitamine," the other not containing nitrogen and stable to alkali treatment would take the name vitasterols (128). The antineuritic vitamin would become vitamine B, and the antipellagra vitamin would be vitamine P. He urges the use of the term vitamin D for the yeast growth-promoting substance, but MacDonald favors the retention of the name bios (215).

To meet the existing confusion the Accessory Food Factors Committee, representing the Lister Institute and the Medical Research Council in England, proposed that the designation vitamin B be used as a blanket term for all dietary factors belonging to this group of water-soluble vitamins; that vitamin B_1 be adopted for Eijkman's thermolabile antineuritic substance; and that vitamin B_2 be applied to the thermostable pellagra preventive of Goldberger and collaborators. The name bios for the substance promoting growth in yeast was left unchanged.[2]

It is clear that the criteria governing a system of nomenclature for the vitamins should involve two features: (a), each vitamin principle essential to the rat should be recognized as a separate entity of equal rank, and each should receive a separate name (no attempt should be made to form an artificial complex by subordinating "components" to a generic name); (b), provision should be made for vitamins which may be discovered in the future. The terminologies set forth by Goldberger *et al.*, Salmon, and Chick and Roscoe are open to criticism in that they

[2] Personal communication from Dr. Chick.

take the misleading step of supplying the complex with a name, implying a subordinate rôle for each factor of the complex. The recommendations of the Accessory Factor Committee show even more subordination of the independent substances, not only in assigning a generic name to the complex but also in giving sub-numerals to constituent factors. Sherman and Axtmayer recognized the need of avoiding subordination, and of placing the substances upon the level of individual vitamins. Actually in using the terms F and G they defeat their expressed purpose, since this leaves vitamin B as a gap or as the "complex" composed of vitamins F and G. The latter is actually subordination.

The physiological results ascribed in the past to vitamin B were probably the composite of several separate effects. Now that a specific action and chemical property, thermostability, have been differentiated from the long accepted characteristics of vitamin B, there is need of names for two recognized factors, each of which is an independent vitamin. This need is met by the McCollum-Drummond system of nomenclature, in which each vitamin is regarded as an entity without class grouping. Under this plan the designation vitamin B would be retained for that part of the original vitamin characterized by antineuritic properties and thermolability. Vitamin B would thus become the antineuritic substance discovered by Eijkman; the term would merely receive a more restricted meaning than it has had during the past decade. Vitamin F, the next available letter, would be assigned to the thermostable substance with characteristic antipellagrous activity, described by Goldberger and his associates. No generic name would be given to this class of dietary factors associated in the water-soluble group; indeed the mention of a complex might well be abandoned.

The complications arising from vitamin B nomenclature may be readily appreciated upon examination of table 1, in which are listed the various names given to the original substance and its recent constituent parts. Throughout this paper the term vitamin B will be used for the substances effecting cures of polyneuritis and promoting growth in rats, except in the discussion involving its multiple nature, when the proper distinguishing names will be applied to distinctive factors.

TEST METHODS. *Animal.* The isolation of the antineuritic principle has been hampered by the lack of a satisfactory qualitative test. Manifestly it is necessary to follow the presence of the vitamin through the various steps in chemical manipulation. Efforts devoted to the perfection of a rapid but reliable method for detecting this dietary factor have yielded indifferent results. The historic test is that which led to the

TABLE 1

Names applied to vitamin B

INVESTIGATOR	SUBSTANCE CURING POLYNEURITIS IN PIGEONS	SUBSTANCE PROMOTING GROWTH IN RAT	THERMOSTABLE SUBSTANCE OR PELLAGRA PREVENTIVE	SUBSTANCE ACCELERATING YEAST GROWTH
Schaumann	Activator			
Funk	Vitamin			
Suzuki *et al.*	Oryzanin			
Tsuzuki	Antiberiberin			
Edie *et al.*	Torulin			
Peters	Torulin*			
Abderhalden and Schaumann	Eutonine	Nutramine†		
Hofmeister	Antineuritin ↓ ↓ Oridin			
McCollum and Kennedy	Water-soluble B	Water-soluble B		
Drummond	Vitamin B	Vitamin B		
Funk; Jansen	Antiberiberi vitamin			
Funk				Vitamin D
Burrows and Jorstad				Archusia‡
Williams, R. J.	Beriberi preventing vitamin	Beriberi preventing vitamin		Beriberi preventing vitamin=Bios
von Euler		hDr		hDm
MacDonald-McCollum	Vitamin B	Vitamin B		Bios
Fulmer-Nelson				
Mitchell	Antineuritic vitamin	Growth promoting vitamin-water-soluble B		

Goldberger *et al.*	Vitamin B $\rightleftharpoons$	Complex: Water-soluble B	$\rightleftharpoons$ Factor P-P	
Salmon	Vitamin B-P $\rightleftharpoons$	Vitamin B	$\rightleftharpoons$ Factor P-P	
Chick and Roscoe	Antineuritic vitamin $\rightleftharpoons$	Water-soluble B vitamin	$\rightleftharpoons$ Vitamin B	
Sherman and Axtmayer	Vitamin F $\rightleftharpoons$	Vitamin B complex	$\rightleftharpoons$ Vitamin G	
Accessory Factor Committee	Vitamin B_1 $\rightleftharpoons$	Vitamin B	$\rightleftharpoons$ Vitamin B_2	Bios
Funk	Vitamin B		Vitamin P (?)	Vitamin D
Authors' suggestion	Vitamin B		Vitamin F	Bios

* Peters uses the name torulin for substance curing head retraction in pigeons; and suggests that it may be different from Jansen's antiberiberi vitamine.

† Abderhalden and Schaumann refer to the unknown substances which make a diet biologically complete. The pigeon was their test animal.

‡ Burrows and Jorstad designated archusia as the non-specific substance necessary not only for growth but also for all activities of a cell. Tissue culture served as a test organism.

discovery of the vitamin; namely, the cure of polyneuritis in animals. Eijkman used fowls in his studies upon the antineuritic vitamin (68); Schaumann extended the list of test subjects to dogs, cats, and monkeys (272). In advancing his claim of isolation of vitamin B in 1912, Funk submitted his product to polyneuritic pigeons (104). In this highly spectacular test the bird after a period on a polished rice or highly purified regimen, is attacked by spastic seizures, characterized by head retraction, "cartwheels," and opisthotonic position. Upon administration of an active preparation the pigeon recovers from its cramps and marvelously gains a normal appearance within a few hours. Almost invariably, however, the "cure" does not persist, and often further attacks cannot be successfully met.

Believing that the pigeon was an unreliable experimental animal, McCollum and Simmonds adopted the rat for testing vitamin B preparations and perfected a technique which has been widely used (223). These investigators pointed to the large number of substances credited with antineuritic properties, because of their reported ameliorative effect upon polyneuritic pigeons. Confusion and doubt were bound to prevail where unrelated materials gave occasional relief to afflicted birds. Accepting the identity of the substance promoting growth in rats and the antineuritic vitamin, they contended that such temporary relief from paresis by pharmacological action was much less satisfactory as a test than that involving the element of growth in the rat. In their proposed procedure rats are restricted to a diet adequate in all respects for growth except for the absence of a source of vitamin B. On such a ration the animals, after a short period of initial gain, soon decline in weight, suffer loss of muscular control, and ultimately die unless the necessary substance is supplied. If a preparation containing the essential vitamin is administered during the period of decline, there is resumption of growth which is maintained as long as the necessary factor is included in the diet.

This rat test, however, did not find favor with Funk (117). For his purpose he divided animals arbitrarily into two groups upon the basis of purine metabolism: the first included those in which the terminal product was allantoin, the second excreted uric acid. Rats together with monkeys and dogs were members of the first class in which Funk stated polyneuritis could not be produced. He held that the disease appeared only in those living forms excreting uric acid, such as birds and man.

During the intervening years since the pigeon test was first used it has

undergone considerable modification. The curative feature has been supplanted in several laboratories by a preventive procedure involving the protection of birds from polyneuritic symptoms while upon a polished rice diet (357). Then, too, Seidell has devised a method in which measured amounts of a preparation under examination are added to a vitamin B deficient diet (292). Presence or absence of the vitamin is indicated by a comparison of the weight curves of pigeons receiving the test material with those of control birds receiving none. The amount required to supply the deficiency in the diet is an index to the potency of the product. With many investigators, however, the establishment of cures remains the favorite test and there have been manifold suggestions bearing upon this technique (190), particularly the removal of such a disturbing element as spontaneous recovery (191). Criticizing both preventive methods, Peters states that each presents a difficulty; the one, the variable time at which convulsions appear in different pigeons on the same diet; the other, the unfounded assumption of the identity of the curative and growth substances in the experiments involving weight (254). In his work he has adhered to the curative test, and he has endeavored to overcome its objectionable features, arising from temporary cures and refractory birds (187a).

Using both the curative pigeon method and the rat growth test in parallel upon a series of vitamin B preparations, Laird described the interesting observation that some preparations giving positive tests with rats were negative with pigeons, and *vice versa* (196). In the light of Plimmer and Rosedale's finding that the vitamin B requirement of pigeons was the smallest of any of the birds studied (255), Laird suggested that in instances where the test with pigeons was positive but the test with rats was negative it was possible that the amount of vitamin B in the preparation was too small for the latter animals. No explanation, however, for the positive results with the rat and negative with the pigeon could be furnished on this basis. He felt that the evidence was too inconclusive to be interpreted in favor of a multiple nature for the dietary substance. Following Goldberger's demonstration of two factors associated in the vitamin, Seidell has shown that the requirements of the two species are quite different (297). He believes that the nutritive needs of the pigeon are the simpler in that it apparently requires only a part of the commonly accepted vitamin B, namely, the antineuritic component, while the rat requires both this component and the thermostable element.

Yeast. It is apparent that a satisfactory test for vitamin B must be

specific, but it must consume neither too much effort, material, nor time. Upon the latter three scores animal tests have met with serious objections. The preparation of diets is laborious at best; in them must be placed a considerable amount of the vitamin fraction under investigation; then weeks must elapse before the animal returns its verdict upon the activity of the material. This is a serious handicap which has beset investigators undertaking the isolation of this vitamin. It is not surprising that from time to time workers in this field have endeavored to evolve a more convenient procedure. Among the proposals has been Williams' yeast test, based upon the assumption that vitamin B, present so abundantly in yeast,* was necessary for the nutrition of yeast cells (349). As early as 1901 Wildiers had noted the great acceleration in growth of yeast cells when an aqueous extract of yeast was added to the culture medium. This effect he attributed to a substance in the yeast extract which he called bios. When Williams found that water extracts known to contain vitamin B likewise stimulated yeast growth, he concluded that bios and vitamin B were identical. The similar behavior of the two substances toward physical and chemical treatment heightened this opinion.

In the apparent requirement of vitamin B for yeast growth Williams saw the possibility of perfecting a qualitative test for the vitamin, or even a quantitative method under standardized conditions. He gathered evidence pointing to a direct relationship between the number of yeast cells appearing in a given time and the vitamin content of materials placed in the medium. His technique consisted in adding an extract, which was to be tested for its vitamin activity, to hanging drops in which were suspended single yeast cells. The rate of multiplication, as followed under the microscope, was an index to vitamin activity. This method was soon superseded by various modifications, among them Bachmann's fermentation test involving the amount of CO_2 produced (11); Williams' gravimetric technique in which the proliferated yeast was weighed directly in a Gooch crucible (350); Eddy and Stevenson's cell count after incubation in sealed tubes (62); and Funk and Dubin's estimation of yeast volume by centrifugalization in specially calibrated tubes (121). For rapidity and ease of performance and for economy of material the yeast test gave great promise. Much enthusiasm was evinced over the prospect of not only detecting the presence but also measuring the amount of vitamin B in foodstuffs and preparations.

After futile attempts, however, at the application of the yeast organism, Souza and McCollum concluded that the test was compli-

cated by so many factors as to make it of little, if any, value (309). It soon became obvious from the work of MacDonald and McCollum (210), and Fulmer, Nelson, and Sherwood (97, 99) that its apparent advantages were completely offset by its lack of specificity. These investigators did not deny that yeast growth could be stimulated by unknown substances; they did hold that these accelerants were not vitamin B but bios, a separate and distinct factor (99, 213). This left, they maintained, the method without value for vitamin B. Sherman and Edgeworth gave such intimation, but they were not so positive in their stand (298). Upon examination of all the suggested yeast tests they placed most confidence in Williams' gravimetric method; but when they compared this with the rat test they stated that the latter was the preferable method. Eventually Eddy and his associates, and Funk and Dubin, the principal advocates of the yeast method, agreed that it was unreliable. The former stated that the test as conducted was not specific for vitamin B, but that this substance was not ruled out as one of the factors in the stimulation (65). The latter authors took the position that the yeast growth-promoting substance, bios, which they called "vitamine D," was separate from vitamin B (123). Later they postulated a relationship between the two factors by claiming that "D" in addition to vitamin B was necessary for growth in rats (122). With these statements the ardor for the yeast method dissolved, and investigators were thrown upon other resources.

Just recently Sweetman and Palmer have proposed a test for vitamin B which is reminiscent of the bios method in that it is based upon the nutritional requirements of a low form of life, the insect (325a). Feeding the confused flour beetle, *Tribolium confusum* Duval, upon a basal ration devoid of vitamin B, they determined the decrease in the larval period which could be attributed to various dosages of vitamin B supplement. Over a certain range of proportions the insect seemed to respond in a quantitative manner to the amounts of vitamin B; further, *Tribolium* was sensitive to such a small amount of the growth essential as 0.5 per cent of a source. In regard to the specificity of the test, the distribution of the growth-promoting substance necessary to the beetle was similar to that of vitamin B, and purified concentrates of the vitamin, prepared by chemical means, were highly effective in producing pupae. Whether evaluation of this test will lead to such a digression as was occasioned by the bios question, is not apparent at this time.

Color reagents. Few color tests are specific for an individual compound; rather they are tests common to a group. This apparent

defect, however, is always advantageous in organic analysis since it gives a hint to the nature of the unknown compound by placing it in its proper class. In addition, a dependable color test has points of superiority over any method involving animal experimentation. A valid test for vitamin B would be that color reaction always given by the vitamin and never by any other substance. At the expense of anticipation it may be said that a satisfactory color test for this factor has never been realized. Some interesting reactions have been observed, nevertheless, in connection with extracts and preparations known to contain vitamin B.

From the vitamin fraction in yeast Funk in 1913 obtained a crude crystalline product which gave a pronounced blue coloration with the uric acid and phenol reagents of Folin and Denis (109). During further fractionation and purification of the product, the reaction gradually disappeared and at the same time the curative action correspondingly diminished. The uric acid reaction depends upon the reduction of phosphotungstic acid in the presence of alkali (88, 89). This is accomplished by numerous substances besides uric acid; among them certain phenols. Peculiarly, this uric acid reagent reacts positively with polyphenols, but does not react with monohydric phenols or their derivatives (89). The highly delicate reagent for phenols, namely, phosphotungstic-phosphomolybdic acid, imparts a blue color with phenols regardless of the number of hydroxyl groups.

Further study of these tests by Funk and Macallum showed that both reactions were given by alcoholic extracts of different foodstuffs, especially those known to be rich in vitamin B (110). Seeking an inkling of the chemical identity of these reacting food substances, which might have antineuritic importance, the investigators applied the color reactions to numerous known compounds. The uric acid reagent, which is much the more limited in its action, gave strong reactions with certain purine and tyrosine derivatives. In this connection Lewis and Nicolet assert that the presence of sulfur replacing oxygen in the 2-position of a purine, pyrimidine, or hydantoin leads to a positive reaction with both reagents (208).

Nothing definite can be said concerning the value of the uric acid color test for vitamin B, although Eddy at one time proposed it as a practical method for standardizing vitamin dosage (59). As was shown by Seidell the water-soluble factor is adsorbed by Lloyd's reagent (286); according to Eddy the uric acid reaction is given by substances adsorbable on this brand of earth (59). Recently Eijkman tested the

crystals claimed by Jansen and Donath to be the isolated antineuritic principle, and he obtained a positive reaction with the reagent (76). Reliability in this test is, however, considerably shaken by the list of too numerous substances which respond with the characteristic color.

Several years ago Jendrassik attempted to find a specific chemical method for detecting the vitamin (179). His work was done with unrelated plant materials, some containing the dietary factor, others free from it. Both were treated with solvents dissolving the vitamin and those not removing it. In this way it was hoped that the antineuritic principle would not be contaminated with the same chemical substances, a condition that might induce a misleading interpretation. Of the tests applied to these extracts he found that the reduction of ferric ferricyanide with the formation of a blue color (Prussian blue) was always given when vitamin B was present and never when it was missing. In no instance was this reaction given by an extract secured from a food containing vitamin B, when the solvent used was known not to remove it; or by an extract obtained from a food not containing the vitamin, although the solvents used extract it when it is present. According to Jendrassik, this strongly suggested that the substance reducing the ferric ferricyanide was vitamin B. Admittedly the reaction was not new; it had been long in use for detecting certain alkaloids.

Incidental to his design of the color test, Jendrassik concluded that vitamin B could not be a phenol since several of the active preparations failed to give phenol reactions (Millon's, Liebermann's). This opinion was challenged by Bezssonoff who stated that a negative result with Millon's tests was not significant inasmuch as this reagent reacts only with typical monophenols (16). He showed further that Jendrassik's test for the vitamin was a very sensitive reaction also for a few o- and p- polyphenols; accordingly a polyphenolic nature for vitamin B was not excluded. A similar view was reached by Levine after application of the Jendrassik reagent to an extensive series of phenolic and non-phenolic compounds (206). His attempt to differentiate between vitamin B and phenols by use of the color test after their destruction by alkali met with no success. Unwittingly Levine failed to mention Bezssonoff's work. This drew from the latter the claim of priority to the possible phenolic nature of vitamin B, and the non-specificity of Jendrassik's reagent for that substance (17). In reply Levine pointed out that Bezssonoff's conclusions were based upon rather scanty evidence from four phenols; Levine had investigated fifty-seven phenols, representing all types (207).

All attempts to perfect an easy, rapid, and economical test for vitamin

B, whether by use of microörganisms or chemical reactions with distinctive colors, have failed. This has been invariably due to lack of specificity. When it is reflected that specificity is the predominant concern in any test, it is readily seen that vitamin investigators have been compelled in the interest of reliability to cling to the animal test, despite its acknowledged disadvantages.

PROPERTIES. Considerable knowledge has been acquired concerning the behavior of vitamin B under various conditions. The many attempts towards the isolation of the substance, although they have failed their objective, have contributed much in the way of its physical and chemical conduct. In turn these facts have proved helpful, particularly in emphasizing necessary precautions which must be exercised if subsequent investigations are to meet with success. No small part has been played by physical chemistry in the study of the properties of the vitamin. While enumerating the applications of this rapidly expanding physico-chemical science, LaMer pointed out that its ramifications extend into a study of the vitamin's solubility, adsorption, dialyzability, and stability (197). The last property has received much attention, especially where heat, acids, alkalies, oxidation, reduction, and radiant energy are concerned. In such investigations time, temperature, and hydrogen ion concentration have been the independent variables, and the importance of their change has been closely followed. For convenience the results from studies on properties have been separated according to the test animals, birds and mammals. Although the results obtained in the earlier work by both methods have been in the main concordant, where discrepancies exist they may find interpretation in the recent belief that the vitamin requirements of these two species are quite different.

Solubility. The very nature of the name water-soluble B, once applied to the vitamin, denotes that it is soluble in water. This was first learned by Eijkman in 1906 while he was attempting to isolate the protective substance from the "Silberhautchen" of rice (71). He further found that it was not precipitated by a moderate amount of alcohol (71, 72). In fact, Fraser and Stanton added increasing amounts of alcohol to an aqueous extract of rice polishings until the solution contained about 91 per cent alcohol by volume without precipitating the active substance (94). It was also early shown by direct extraction method that the antineuritic principle was soluble in strong alcohol (93, 72).

In his separation of an antineuritic product Funk extracted rice

polishings with alcohol containing hydrogen chloride (104). In subsequent work he asserted that alcohol was a poor solvent for the vitamin since repeated extractions of yeast with it removed only a small part of the substance, leaving the residue extremely active (106). This claim was substantiated by Vedder and Williams, who found that three successive extractions with cold 95 per cent alcohol failed to remove all of the antineuritic principle from rice polishings (336). They stated that 90 per cent alcohol was a much better solvent. Penau and Simonnet described similar incomplete extraction with alcohol, and added the interesting observation that a cold alcoholic extract was superior to a warm one (252).

Results quite different from these were reported by Cooper who maintained that absolute alcohol readily extracted the vitamin from meat (32, 33). Furthermore, extraction of rice bran with 0.3 per cent hydrochloric acid, 70 per cent alcohol, or 96 per cent alcohol containing hydrogen chloride gave the same result, namely, the vitamin was quantitatively removed from rice bran, according to Jansen (175). Several workers observed that the vitamin was insoluble in ether (26, 32, 33, 317). McCollum and Kennedy stated that acetone, benzene, and ethyl acetate removed the antineuritic vitamin from ether-extracted wheat germ (220). Steenbock confirmed its solubility in acetone (312), although Abderhalden and Schaumann employed this as a precipitant in the preparation of "eutonine" (3). Using autolyzed yeast filtrate as the source of vitamin B, Myers and Voegtlin found that olive oil and oleic acid removed the active principle, showing, they claimed, that it was fat-soluble as well as water-soluble in the form of a crude extract (235). In all these experiments pigeons, fowls, or cocks suffering from polyneuritis were used as the test animals.

Coincident with their discovery that wheat germ and rice polishings contained a substance essential to growth in rats, McCollum and Davis mentioned that it was soluble in water (217). Osborne and Mendel, and Drummond soon noted that this vitamin, which promoted growth, was insoluble in strong alcohol (240, 48). The latter likewise reported its insolubility in ether (48). In a study of the solubility of vitamin B in organic solvents, McCollum and Simmonds found that it was not extracted directly from beans or wheat germ by ether, benzene, or acetone, but was in great part by alcohol (223). Peculiarly, when the active substance was extracted by alcohol it then became soluble in benzene. Williams and Waterman later explained this phenomenon by showing that McCollum and Simmonds had actually used a low boiling

ternary mixture of alcohol 18.5 per cent, water 7.4 per cent, and benzene 74.1 per cent, in which the vitamin was found to be soluble (361). Levine, McCollum, and Simmonds described glacial acetic acid as an excellent solvent for vitamin B, but there is no certainty that moisture was entirely excluded from the plant materials used in the experiment (205).

In search of a solvent that would give a potent extract and at the same time carry a minimum of contaminating substances, Funk, Harrow, and Paton investigated the ability of ethyl alcohol (50, 60, 70, and 80 per cent), methyl alcohol (60 and 70 per cent), propyl alcohol, butyl alcohol, isobutyl alcohol, acetone, methylethylketone, and acetic acid, all 70 per cent strength, to remove the vitamin from yeast (127). With two criteria in mind they tested the extracts and residues on both pigeons and rats. When the standard was not only activity of extract but also inactivity of residue, the most successful solvent was 70 per cent ethyl alcohol. When inactivity of residue was neglected and activity of extract with minimum of impurities was the standard, acetone was the best solvent.

McCollum and Kruse found that a 95 per cent alcoholic extract of wheat germ contained little vitamin B (225). However, when such extracts were prepared by use of 95 per cent alcohol containing 1 per cent gallic or tannic acid, excellent responses were produced in rats. They interpreted this to indicate that the solubility of the vitamin in the form of a salt was evidently greater in alcohol than was the vitamin itself. Incidentally this union with an acid strengthened the view that the vitamin was of basic nature. Other acids yielded results varying from good to negative. By this method they also tested the possibility of forming alcohol-soluble compounds of certain double salts of which the vitamin might be a component. Zinc and cadmium chlorides in alcohol yielded extracts decidedly more active than was to be expected from the solvent without the salts.

Dialyzability. Among the properties of the antineuritic principle described by Eijkman was its dialyzability (71). Using a parchment membrane Chamberlain and Vedder confirmed this observation, and drew the conclusion that the active factor existed in a crystalloid condition; thus, eliminating all substances in a colloidal state (24). The same experiment has been repeated by Shiga and Kusama (304), Drummond (48), and Seidell (291). If an air dried collodion membrane, which is highly impermeable, is placed in solutions of alcohol and water, it becomes permeable and the permeability increases with the strength of

the alcohol employed. Zilva and Miura used such a calibrated membrane in their studies on the diffusibility of vitamin B (365). They found that the active principle would not penetrate a sac treated with alcohol of 90 per cent or lower concentration, but that it diffused entirely through a 95 per cent or 100 per cent membrane. In comparison with known organic compounds, the vitamin moved through a membrane of such permeability as permits the passage of substances like methylene blue, neutral red, and safranine. They suggested that the active principle may be in a semicolloid condition.

Stability. Heat. Grïjns was the first to note that heating to 120°C. for one hour destroyed the antineuritic activity of katjang idjo (139). This experiment has since been repeated many times at various temperatures and different periods of exposure and the results with few exceptions have uniformly been in accord. In most instances these repetitions have been prompted by the practical aspect of determining the factors of safety, regarding temperature and time, in heating foodstuffs. Nevertheless, these studies on vitamin destruction by high temperature have had a scientific application in the attempts at isolating vitamin B. In table 2 are listed the results reported by many investigators using birds, cats or dogs as test animals.

There are technical difficulties concerned in the study of high temperature destruction of vitamin B, and these have not always been fully appreciated. Fraser and Stanton noted that unpolished rice placed in a basin or suspended in a small bag in the autoclave suffered vitamin impairment at 120° for one hour (94). When the rice was immersed in water during the autoclaving at the same temperature and for the same period, there was no reduction in antineuritic activity. They concluded that some physical condition other than temperature influenced the result, since immersion in water seemed to negative entirely the destructive effect of the atmosphere of steam in the autoclave. Whatever variable results have been obtained by different investigators in this work is doubtless due, among other things, to failure in control of experiments. Almost without exception the autoclave has been used to attain high temperatures. It is obvious that the temperature calculated from the pressure gauge may not coincide with the actual temperature applied to the foodstuffs, as indicated by a maximum recording thermometer placed centrally within the material. The nature of the substance, the state of its division, and the size of the vessel containing it may contribute to this discrepancy.

Chick and Hume were aware of these pitfalls, and they carefully

TABLE 2

Thermal destruction of vitamin B

INVESTIGATOR	VITAMIN SOURCE	AUTOCLAVE TEMPERATURE	EXPOSURE TIME	RESULT	TEST ANIMAL
		°C.			
Grijns (139)	Katjang idjo beans	120	1 hour	Destruction	Fowl
Eijkman (71)	Unmilled rice	115–125	2 hours	Destruction	Fowl
Holst (164)	Beef	110–120	1 hour	Destruction	Fowl
	Beef	100	½ hour	No destruction	Fowl
	Peas	120	½ hour	No destruction	Fowl
Fraser and Stanton (94)	Unpolished rice	120	1 hour	Destruction	Fowl
Shiga and Kusama (304)	Rice bran	130		Destruction	Fowl
Barsickow (13)	Yeast	120		No destruction	Pigeon
Chick and Hume (28)	Yeast	100	Prolonged	Slow impairment	Pigeon
	Wheat germ extract	120	2 hours	Rapid impairment	Pigeon
Chick and Hume (29)	Wheat germ	100	2 hours	No destruction	Pigeon
		113	40 minutes	50% destruction	Pigeon
		118–124	2 hours	75% + destruction	Pigeon
Guerrini (144)	Rice	150	3 hours	Destruction	Pigeon
Weill and Mouriquand (343)	Barley	120	½ hour	Destruction	Pigeon
Portier and Randoin (257)	Grain	124–128	1 hour	Destruction	Pigeon
Nitzescu (237)	Grain	110–120	1 hour	Destruction	Pigeon

TABLE 2—*Concluded*

INVESTIGATOR	VITAMIN SOURCE	AUTOCLAVE TEMPERATURE	EXPOSURE TIME	RESULT	TEST ANIMAL
		°C.			
Emmett and Luros (80)	Unmilled rice	120	2–6 hours	Total destruction	Pigeon
Davis (44)	Grain	120	2½ hours	Destruction	Pigeon
Weill, Mouriquand and Michel (344)	Beef	120	1½ hours	Destruction	Cat
Voegtlin and Lake (338)	Beef	120	3 hours	Incomplete destruction	Cat, dog
Karr (181)	Yeast	120	3–4 hours	Partial destruction	Dog

recorded the internal temperature (29). Portier and Randoin found that finely cut vegetables, when heated to 130°C. as indicated by the autoclave manometer, actually had a temperature of only 98° to 115° as recorded by a maximum thermometer fixed in the center of the food (257). With grains the central temperature fluctuated between 97° to 106°. Even when the latter were placed in small vessels the central temperature seldom reached and never exceeded 120°, but when they were suspended in a bag in the middle of the autoclave, the central temperature was only one or two degrees lower than the vapor temperature indicated by the manometer.

Nitzescu found that the antineuritic principle in wheat and maize was destroyed by autoclaving for one hour at 110° to 120°C.; if, however, these grains were heated in closed bottles in the autoclave, with other conditions the same, the vitamin was not destroyed (237). Similarly, heating these materials at the same temperature for even a longer time in a drying oven was without effect upon vitamin B. He concluded that the destruction of the vitamin was caused by steam at high temperature. Scotti-Foglieni likewise showed that the antineuritic principle in rice is not destroyed by autoclaving between 120 to 134°, if it is contained in hermetically sealed vessels (281).

Fewer experiments have been conducted upon the destruction of vitamin B at high temperatures with the rat as the test animal. In

TABLE 3

Thermal destruction of vitamin B

INVESTIGATOR	VITAMIN SOURCE	AUTOCLAVE TEMPERATURE	EXPOSURE TIME	RESULT	TEST ANIMAL
		°C.			
McCollum and Davis (219)	Wheat embryo	120	1 hour	No destruction	Rat
	Skim milk powder	120	1 hour	Destruction	Rat
McCollum, Simmonds, Pitz (221)	White beans	120	1¼ hours	No destruction	Rat
Drummond (48)	Yeast extracts	100	30 min.	No destruction	Rat
		120	30 min.	Incomplete destruction	Rat
Daniels and Nichols (40)	Soy bean in water	120	30–40 min.	No destruction	Rat
Daniels and McClurg (41)	Navy bean Soy bean	120	40 min.	No destruction	Rat
McCollum, Simmonds and Parsons (224)	Pea	120	1¼ hours	No destruction	Rat
Emmett and Luros (80)	Unmilled rice	120	2–6 hours	No destruction	Rat
Sherman and Spohn (299)	Skimmed milk powder dry	100	48 hours	No destruction	Rat
	Skimmed milk powder fluid	100	6 hours	25% destruction	Rat
Sherman and Grose (300)	Tomato juice	100	4 hours	20% destruction	Rat
		110	4 hours	33% destruction	Rat
		120	4 hours	45% destruction	Rat
		130	4 hours	55% destruction	Rat
Goldberger *et al.* (136)	Yeast	120	2½ hours	Destruction	Rat

table 3 it is seen that practically all investigators, except Drummond, Sherman and associates, and Goldberger *et al.*, report that high temperature produces no apparent destruction of the vitamin essential for growth in rats. A possible explanation lies in the high levels at which the autoclaved material was fed; in consequence, even considerable destruction of the vitamin would leave a margin of safety, and would not be detected. Sherman and Grose contend that the heat destruction of vitamin B proceeds measurably at 100°C and at an increasing rate as the temperature is raised (300). They found no evidence of departure from the orderly course of a chemical reaction under the accelerating influence of heat, although the rate of vitamin destruction was slightly lower than that for most reactions. Their conclusions were based upon the reduction in vitamin activity of tomato juice after four hours at pH 4.3 and at the respective temperatures of 100°, 110°, 120°, 130°C.

It is now accepted that the antineuritic properties of vitamin B are destroyed at temperatures of 115 to 120° for one to two hours, a loss which manifests itself not only in the pigeon but also in the rat. Deterioration at lower temperatures is much slower; indeed the bulk of evidence tends to indicate that heating at the temperature and for the length of time ordinarily employed in cooking, namely 100°C., produces insignificant destruction of vitamin B. Despite Randoin and Lecoq's assertion that yeast dried in the air at 50°C. suffered a loss in antineuritic effect (260), the presence of the vitamin in dry grains is evidence that it can withstand desiccation.

Acids. That vitamin B is remarkably resistant to acid hydrolysis has been recognized for some time. Cooper and Funk in 1911 noticed that yeast heated with 20 per cent sulfuric acid for 24 hours still retained its curative properties for pigeons (31). Shortly thereafter Shiga and Kusama reported that the vitamin withstood boiling with 0.5 per cent hydrochloric acid (304). Indeed investigators came to hold the view that acid hydrolysis was required to liberate the substance from its natural bound condition. Believing in this wise that strong mineral acids split the antineuritic principle in rice polishings, thereby reducing it to a more active as well as simpler form, Vedder and Williams hydrolyzed polishings with 50 per cent sulfuric acid (336). They claimed that this procedure permitted the later separation of an active fraction not obtainable from unhydrolyzed polishings. Similarly Sullivan and Voegtlin stated that a crude lipoid fraction obtained by 95 per cent alcoholic extraction of wheat bran possessed little, if any, antineuritic effect. After acid hydrolysis, however, the fraction yielded a vitamin

preparation which manifested strong activity (317). Although acid hydrolysis is no longer regarded as necessary in the preparation of active extracts or fractions, the resistance of the antineuritic principle to boiling acids has been repeatedly demonstrated (312). Drummond asserted that the vitamin activity of "marmite," a commerical yeast product, was not greatly diminished after boiling for twelve hours with 1 per cent hydrochloric acid (48). When hydrolyzed at boiling temperature for ten hours with 20 per cent sulfuric acid, the preparation showed greatly decreased power to promote growth in rats. He believed that this was due not so much to actual destruction as to adsorption of the vitamin on barium sulphate during the removal of the acid by baryta. This view is likely, inasmuch as McCollum and Simmonds found that the vitamin possessed great stability toward hydrochloric acid (223)

Alkalies. Contrary to its marked resistance to acids, vitamin B is extremely sensitive to an alkaline reaction, particularly with the application of heat. As early as 1909 Schaumann produced polyneuritis in pigeons, cats, and dogs by feeding them horse muscle which had been heated for two or three hours at 120°C. in the presence of 20 per cent sodium hydroxide (271, 272). He attributed this result to destruction of nucleo-proteins which he then believed to constitute the antineuritic principle. Since the harmful effect of high temperature upon the vitamin was then known, it was not clear at that time whether the strong alkali contributed to the deterioration of the antineuritic principle or whether the heat was the sole agent. The latter alternative seemed likely when Shiga and Kusama reported that the vitamin was not destroyed by heating at 100°C. in 1 per cent sodium hydroxide (304).

During attempts to isolate the active principle from an alcoholic extract of rice polishings by precipitation with sodium hydroxide, added to the extent of 0.3 per cent, Fraser and Stanton found that both the precipitate and filtrate were inactive (94, 95). They were led to infer that the vitamin either was separated into two parts or was decomposed by weak alkali. In line with the latter thought, Chamberlain, Vedder and Williams stated that treatment of an antineuritic extract with sodium hydroxide did destroy its activity, but they furnished no details on this point (26). Again Vedder and Williams were inclined to attribute their failure in isolating the vitamin to the alkali treatment used in the procedure, but again their suggestion was not supported by evidence (334). In a subsequent paper these authors reported that strongly alkaline reagents destroyed the antineuritic principle (336). Despite these intimations, the destructive effect of alkali upon vitamin B was

first clearly demonstrated by Cooper (33). Upon adding ammonia to a vitamin solution of known potency until the concentration reached 10 per cent, and then keeping the mixture in a closed flask at room temperature for twenty-four hours, he found that 50 per cent of the antineuritic activity was lost by this contact with alkali. This was not confirmed by Williams and Seidell who permitted an autolyzed yeast filtrate solution containing 10 per cent sodium hydroxide by weight to stand at room temperature for five hours (356). They found that the curative properties of the preparation had suffered little destruction. However, the behavior of "activated" fullers' earth under similar treatment led them to qualify their previous statement, and to conclude that "the activity of the constituents of autolyzed yeast adsorbed by fullers' earth is a combination of antineuritic and weight-maintaining functions, and it is the latter only which is materially affected by alkalies under the conditions of the present experiments."

The destructive effect of alkalies has since been substantiated by numerous workers, as is shown in table 4, but the results have presented some inconsistencies.

When examined in the light of three variable factors, temperature, exposure time, and alkali concentration, the apparent discrepancies in the data upon alkali destruction of the vitamin largely disappear. For example, Drummond found that "marmite" treated twenty-four hours at room temperature with 5 per cent sodium hydroxide showed little impairment in its vitamin effect, but that the same preparation heated with the alkali for five hours suffered almost complete loss of activity (48). Even more clarifying is the work of Osborne and Leavenworth, in which the potency of a yeast fraction is reported not to be appreciably affected by solution in 0.1 normal sodium hydroxide for a half hour at 20°C. or for eighteen hours at the same temperature (248). When this preparation, however, stood in the alkali for ninety hours at 20°C., the vitamin activity was seriously impaired. More rapid destruction occurred if the yeast fraction stood in the alkali for eighteen hours at room temperature, followed by heating at 90°C. for one hour.

Much the same results were obtained by Sherman and Burton in experiments in which the degree of alkalinity, in terms of hydrogen ion concentration, and time were the variables (301). Shifting the pH of tomato juice successively to 7.9, 9.2, and 10.9, with an hour's boiling at each pH, produced an increase in vitamin deterioration as shown by the respective percentages of 32, 66, and 90–100. With four hours' boiling at these pH's the respective percentages of destruction became 70, 100, and

TABLE 4

Effect of alkali treatment upon vitamin B

INVESTIGATOR	VITAMIN SOURCE	ALKALI CONCENTRATION	TEMPERATURE (CENTIGRADE)	EXPOSURE TIME	RESULT	TEST ANIMAL
Schaumann (271)	Horse muscle	20% NaOH	120°	2–3 hours	Destruction	Pigeon
Schaumann (272)	Horse muscle	Na_2CO_3	110–120°	1–3 hours	Destruction	Cat, dog
Shiga and Kusama (304)	Rice bran	1% NaOH	100°		No destruction	Monkey
Fraser and Stanton (94, 95)	Rice polishings	0.3% NaOH	Room	Brief	Destruction	Fowls
Cooper (33)	Horse flesh	10% NH_4OH	Room	24 hours	50% destruction	Pigeon
Williams and Seidell (356)	Autolyzed yeast	10% NaOH	Room	5 hours	No destruction	Pigeon
Steenbock (312)	Egg yolk	conc. sol.	Room		No destruction	Pigeon
		dil. sol.	Boiling		Ready destruction	Pigeon
Voegtlin and Lake (338)	Lean beef	10% Na_2CO_3	120°	3 hours	Destruction	Cat, dog
Drummond (48)	Marmite	5% NaOH	Room	24 hours	Little destruction	Rat
		5% NaOH	Hot	5 hours	Destruction	Rat
McCollum and Simmonds (223)	Wheat germ	0.28% NaOH	Hot	1 hour	Destruction almost complete	Rat

Daniels and McClurg (41)	Navy beans	33 cc. 5% $NaHCO_3$	Boiling	90–120 min.	No destruction	Rat
	Soy bean extract	6.4 cc. 0.1 N NaOH	Boiling	30–60 min.	No destruction	Rat
Osborne, Wakeman and Ferry (245)	Yeast	0.1 N NaOH (10 times weight)	Room Boiling	$21\frac{1}{2}$ hours 2 hours	No destruction	Rat
Osborne and Leavenworth (248)	Yeast fraction	0.1 N NaOH	20° 20° 20° Room 90°	$\frac{1}{2}$ hour 18 hours 90 hours 18 hours 1 hour	No destruction No destruction Destruction Destruction	Rat Rat Rat Rat
Sherman and Burton (301)	Canned tomatoes	pH 7.2 pH 7.2 pH 9.2 pH 9.2 pH 10.9 pH 10.9	Boiling Boiling Boiling Boiling Boiling Boiling	1 hour 4 hours 1 hour 4 hours 1 hour 4 hours	32% destruction 70% destruction 66% destruction Total destruction Total destruction Total destruction	Rat Rat Rat Rat Rat Rat

100. These results indicated that not only raising the concentration of hydroxyl ions but also prolonging the period of exposure increased the destruction of the antineuritic principle at boiling temperature.

The really important conclusion regarding the sensitivity of vitamin B to alkalies, in so far as it affects the concentration or isolation of the substance, may be drawn from Osborne and Leavenworth's statement that dilute alkaline solutions can be used without materially affecting antineuritic activity, provided a low temperature and a short exposure are carefully maintained (248).

Diazotization, oxidation and reduction. Believing that an amine nature had been imputed to vitamin B without sufficient evidence, McCollum and Simmonds subjected an active extract of wheat embryo to treatment with nitrous acid, a reagent which diazotizes amines (223). The antineuritic principle suffered little, if any, destruction. They considered this resistance to nitrous acid as strong evidence that the vitamin was neither a primary nor a secondary amine, since diazotization of the substance would scarcely occur without serious disturbance to its physiological activity. Levene and van der Hoeven (204), and Peters (254) confirmed this stability to nitrous acid. Apparently the vitamin is likewise resistant to oxidation in a slightly acid medium. Exposing autolyzed yeast filtrate for six hours to the action of ozone, Zilva found very little destruction of vitamin activity (366). Kramár tested the effect of reduction on vitamin B by subjecting alcoholic extracts of yeast to eight-hour action of zinc-hydrochloric acid in acid medium, and of aluminum amalgam in weakly alkaline reaction (194). The antineuritic factor withstood reduction in acid medium, but it suffered destruction by reduction in the alkaline medium. That the loss of activity in the latter instance was not due to the alkaline reaction was shown by control tests.

Radiant energy. Funk reported that radium emanation did not impair the vitamin B activity of autolyzed yeast (120) but Sugiura and Benedict maintained that exposure to the rays produced a partial inactivation (315). According to Weill and Mouriquand, x-rays exerted no deleterious influence upon the vitamin (345). There is difference of opinion regarding the effect of ultraviolet irradiation of the antineuritic principle. Working independently, Zilva (364) and Špinka (310) agreed that exposure of yeast to ultra-violet rays for twenty minutes at a distance of ten centimeters, or for six hours at a distance of six inches, had no effect upon its vitamin activity. R. R. Williams, however, asserted that the antineuritic factor in an aqueous yeast

extract was completely destroyed by irradiation for a few hours (360). By spectrographic technique Damianovich made the highly interesting observation that an active extract of yeast, although practically colorless, showed a characteristic absorption band between 2478 and 2600 Å, which seemed to correspond to that of pyrimidine compounds (39).

Autolysis and age. Cooper has stated that yeast which has undergone autolysis is as strongly curative as the original material (35). For a time investigators, engaged in separating the active principle from yeast, employed the autolyzed product as their starting material; lately they have inclined to water extracts of the intact cells. Samples of Indian lentils and peas kept for thirty-eight years still contained appreciable quantities of vitamin B as shown by their favorable effect upon rats (86). However, they appeared to have lost a small amount of their vitamin content, according to Findlay. Rice stored as paddy for one hundred years was reported by Jansen to have lost little or none of its antineuritic vitamin (176). The remarkable stability of vitamin B under ordinary conditions would lead *a priori* to the conclusion that it could withstand prolonged preservation.

ATTEMPTS AT ISOLATION AND IDENTIFICATION. At the outset the admission must be made that the isolation of vitamin B has not been achieved and demonstrated to general satisfaction. Assuredly it has not been from lack of effort; upon no substance, except perhaps some of the endocrine principles, has more diligence been lavished. With the burst of enthusiasm over the discovery of vitamins, the number of attempts to separate the antineuritic principle in crystalline form multiplied almost beyond bounds. Unfortunately in many instances zeal overcame discretion, so that the history of chemical research upon vitamin B is a series of premature notices of its isolation which met an early end. They are now exhumed in the interest of completeness and for any new meaning they may gain in the light of present viewpoints. It is true that our knowledge of vitamin B, like its fellow factors, is limited to its physiological effects and to some aspects of its chemical behavior. Its existence has been manifested by its cure of a specific disease and by its promotion of well-being; none the less by its definite response to chemical treatment; but its chemical nature has not been revealed.

If the investigation of vitamin B has not yielded the desired results, its indirect effects have not been barren. While engaged in methods for isolating the antineuritic substance, Hopkins gained a discovery of equal magnitude, the separation of glutathione (166). Somewhat similar

fortune befell several Japanese investigators in the finding of new compounds among the constituents of rice. In their search for vitamin B in yeast extract Suzuki, Odaki and Mori obtained crystals which they later identified as adenylthiomethylpentose (323, 324). From the same source Odaki succeeded in separating a new thio-amino acid (238), and several unknown bases (239). Funk found nicotinic acid in the vitamin fraction of rice polishings (109). Finally, Abderhalden and Schaumann in the course of their concentration of the antineuritic principle discovered a methylated amine, dimethyl-propenyl-amine, to which they gave the name "aschamin" (3). In this way organic chemistry has been enriched by the stimulus from vitamin B research.

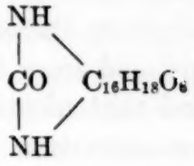
Two principal courses lie open in undertaking the isolation of vitamin B. One procedure involves the gradual separation of inert, contaminating substances from a vitamin-rich extract until the antineuritic factor is left in pure form; the other depends upon the removal of the vitamin directly from the predominating mass of inactive material by specific agents. These measures have been applied in such processes of vitamin isolation as specific chemical precipitation, fractional precipitation by alcohol, and selective adsorption. It may be mentioned that practically all methods employed in vitamin concentration fall naturally within these three classifications. A somewhat unrelated fourth means, which was devised for identification of the antineuritic substance, was the administration of known organic compounds to test animals.

Chemical precipitation. The early chemical work upon the antineuritic factor can again be traced to the Dutch investigators in Java. It was not long after Eijkman and Grĳns' pioneer work in demonstrating the existence of this dietary principle that Hulshoff-Pol claimed its isolation (167). His method involved the precipitation of an aqueous extract of katjang-idjo beans by basic lead acetate. Decomposition of the resulting precipitate yielded his chemically pure decoction, the concentration of which produced 50 to 100 mgm. of crystals of two varieties (168). These dissolved with difficulty in water, giving it a highly acid reaction. From this property the substance was given the name X-acid. Following tests upon polyneuritic fowls Grĳns cast doubt upon the therapeutic value of the preparation. In a tardy effort to vindicate the activity of his crystals Hulshoff-Pol announced ten years later that the X-acid effected cures when properly administered (168). There can be no doubt that whatever activity was possessed by the crystals was due to the vitamin present solely as an impurity. No light was thrown upon the chemical nature of the essential factor by this research, so that

nutrition investigators in 1910 could claim definite knowledge of only a few facts: the active principle's solubility in water, and acid alcohol; its dialyzability; its destructability by heat (113).

In 1911 Cooper and Funk stated that the antineuritic principle was precipitated entirely from an aqueous extract of yeast by phosphotungstic acid, and that it could be separated from the precipitate by baryta (31). The success of this procedure suggested to Funk that the active substance belonged to the class of basic compounds, since phosphotungstic acid is the most general precipitant for them. In the next year he examined an extract of rice polishings systematically by the best known methods of separating nitrogenous bases. As the first step, the curative principle was precipitated as phosphotungstate. When freed from this combination, the vitamin was partially carried down by mercuric chloride; it was more completely precipitated by silver nitrate in the presence of barium hydroxide. By this technique Funk obtained a small crop of crystalline needles which effected cures in polyneuritic pigeons. The crystals melted at 223°C.; they were insoluble in cold water and in alcohol, but were soluble with difficulty in hot water. Reactions showed that the compound was a nitrate of a base, and from combustion analysis Funk assigned the formula $C_{17}H_{18}O_4N$ (HNO_3) to it (104). It was to this substance, organic and basic in nature, that he gave the name "vitamine" (105).

The small yield of the active substance from rice polishings led Funk to turn to yeast as a starting material for subsequent work. When the original method was repeated upon yeast, except for the omission of sublimate, the final silver-baryta fraction yielded the curative crystals melting at 223°C., *i.e.*, the same product that had been isolated from rice polishings (106). At this time Funk corrected his previous statement that the compound was in the form of a nitrate; he now asserted that it was a free base. From the chemical behavior of the active substance he suggested that it was a pyrimidine base, analogous to uracil and thymine; and that it probably was a constituent of nucleic acid. He assigned the hypothetical formula



to it.

In testing this compound isolated from rice polishings and yeast, Funk

found that its antineuritic power was somewhat less than that of the crude fraction from which it was obtained (107). Meanwhile, the administration of known organic substances to polyneuritic birds had shown that a number of purine and pyrimidine derivatives possessed some curative effect (108). Funk was struck with the possibility that besides the compound melting at 223° small quantities of other substances present in the silver-baryta fraction might be necessary for complete antineuritic action, although no such substances had been isolated (107). Making a careful examination of this fraction from yeast, he obtained a crude crystalline product (109) which induced a quick recovery in polyneuritic pigeons, but could not maintain life for more than a few days when added to polished rice (111).

When this crude product was fractionated it yielded three substances (109) which individually or collectively, however, showed no antineuritic action (112). The first compound was a strong acid with the empirical formula $C_{24}H_{19}O_9N_5$, and apparently identical with the substance melting at 223°; the second possessed the formula $C_{29}H_{23}O_9N_5$, and produced a positive reaction with Folin's phenol test; the third was isolated as a picrate, and was identified as nicotinic acid (109). By similar investigation of the vitamin fraction from rice-polishings Funk separated two substances: one with the formula $C_{26}H_{20}O_9N_4$, the other was nicotinic acid. It is interesting that the crude product obtained from yeast was curative and gave the Folin uric acid reaction; but the three substances resulting from its fractionation were without antineuritic properties, as mentioned, and did not respond to the color test. Apparently the substance or substances responsible for curative activity and the color reaction had disappeared during the process of purification. Funk hints at a possible destruction during chemical manipulation, since the isolation of nicotinic acid in the active fraction from two sources led him to mention that it was possibly a decomposition product of the vitamin.

Repeating the investigation of rice polishings, Funk in conjunction with Drummond found that the previously isolated substance with the formula $C_{26}H_{20}O_9N_4$, was really nicotinic acid (47), as Barger had suggested (12). These investigators, therefore, were not able to isolate any substance other than this acid from the vitamin fraction of rice polishings, and they concluded that nicotinic acid had contained the vitamin in some cases as a contamination. In a statement that withdrew all of his previous claims of success Funk at this point asserted: "We have . . . . failed to isolate the curative substance which occurs

in rice polishings. It is apparently decomposed during the fractionation and all trace is lost."

Although Funk had not secured the vitamin in pure form, his efforts in this direction were unabated. His crude vitamin fractions, concededly active in curing polyneuritis in pigeons, had been criticized as unable to maintain weight and life in birds on polished rice, a property possessed by the material from which they were extracted (111). Facing this new angle, he announced in 1916 that he had obtained from yeast a vitamin fraction whose action was complete in that it manifested at once antineuritic and weight-maintaining properties (112). The preparation comprised the acetone insoluble fraction of the phosphotungstate precipitate. When this was examined further, however, it was found to consist mainly of adenine, so that again the separation of the vitamin in a crystalline state was unachieved.

After an attempt to substantiate Funk's work, Chamberlain, Vedder and Williams reported that they were unable to precipitate the active principle from rice polishings by phosphotungstic acid (26). At the same time they suggested that their failure might have been due to destruction of the vitamin in the baryta decomposition or in precipitation by basic lead acetate, which they had employed in the procedure. A second trial, in which Funk's plan was followed more closely, was still unavailing; but they did not regard this ineffectual effort as evidence against his work, inasmuch as they had introduced some slight modifications (334). Finally, by initial hydrolysis of the active extract, they were able to confirm the precipitation of the curative substance (336). The use of phosphotungstic acid and silver nitrate-baryta in the chemical investigation of vitamin B, as proposed by Funk, has been repeated many times, but with no more success than was recorded by him (48, 85, 173, 316, 346, 354).

Simultaneous with Funk's publication on the "isolation" of the antineuritic vitamin appeared similar papers by Suzuki, Shimumura, and Odake (321); Edie, Evans, Moore, Simpson and Webster (57); and Schaumann (274). Upon apparently valid evidence Suzuki disputes Funk's priority not only in regard to the preparation of an antineuritic concentrate, but also in the laying of the foundation for the modern vitamin theory (325). With respect to Suzuki's first claim it is fitting to recall Schaumann's comment that the defects of all the early chemical investigations on the vitamin have become so apparent that priority is rather an empty honor (278). As for the recognition that unidentified factors were necessary in the diet, and that noxious consequences arose

from their absence, this view was the outgrowth of development rather than the outcome of a single discovery.

In their work upon the separation of the active principle from rice bran, Suzuki and his collaborators likewise employed the reagent, phosphotungstic acid. The resulting precipitate, when decomposed, yielded a yellow-brown syrup, "rohoryzanin I," which hardened upon drying (320). Among the cleavage products of rohoryzanin I after hydrolysis were choline, nicotinic acid, glucose, and two acids; α-acid with the formula $C_{10}H_8NO_4$, and β-acid with the formula $C_{18}H_{16}N_2O_9$. From rohoryzanin I was prepared rohoryzanin II by precipitation with tannin. When rohoryzanin II was treated with picric acid, the active principle separated as oryzanin picrate (321). These authors calculated that 5 mgm. pure oryzanin were just as active as 3 grams rice bran, a relation indicating an extremely potent preparation.

Working upon the structural chemistry of β-acid, Sahashi has shown that in all probability it is 2.6-dioxyquinoline-4-carboxylic acid (266), containing one molecule of water of crystallization (265). Recently he has reported that the acid has a certain antineuritic power, and he has concluded that the nucleus of the antineuritic factor of oryzanin would consist either of the 2.6-dioxyquinoline group or its tautomeric form (267). That Suzuki has not contributed additional information upon his pure oryzanin is unexplainable. Drummond and Funk have been unable to confirm his findings; they neither detected the α- and β-acids, nor obtained the curative oryzanin picrate (47).

After experimentation with numerous methods for separating the active substance, Edie, Evans, Moore, Simpson and Webster finally adopted a procedure in which yeast was extracted with alcohol; the solution was cleared with basic lead acetate; and precipitation was effected with silver nitrate-baryta (57). Upon decomposition the precipitate yielded a small amount of crystalline organic material contaminated with ash, principally barium nitrate and phosphate. This residue, to which they gave the name torulin, showed a formula of $C_7H_{16}NO_2(HNO_3)$. The presence of an amine group in it was indicated by the detection of trimethylamine upon baryta treatment. Unfortunately the activity of torulin was not determined.

At the same time that Funk, Suzuki and associates, and Edie *et al.* had been describing the isolation of the antineuritic principle, Schaumann had succeeded in separating several active substances from rice bran. Two preparations, obtained by different methods, were described as essential for protection from polyneuritis (273). The first was a phos-

phatid containing a carbohydrate; the second was a mixture of carbohydrates containing phosphorus. Both substances not only prevented the occurrence of polyneuritis in pigeons fed white rice, but also in proper dosage increased the weight of the birds. Later Schaumann isolated a nitrogenous compound free from phosphorus (274). He called this "activator." His method involved the removal of impurities from an aqueous extract of rice bran by alcohol; and precipitation of the active substance by mercuric chloride. That part of the precipitate which proved soluble in water was decomposed, and the liberated principle was precipitated by phosphotungstic acid. Decomposition of the phosphotungstate by baryta yielded crystalline needles, of which 0.2 gram cured paralyzed pigeons.

From his paper it is apparent that Schaumann did not wish to state that the cure of polyneuritis depended exclusively upon this substance. Rather he inclined to the view that this isolated base played an intermediate rôle. Since very small amounts of the "activator" were sufficient to save animals, and its effect was prolonged, Schaumann attributed a ferment-like action to it; perhaps as a phosphotase. The active substance was found almost exclusively in the pericarp of rice seed, the main depot for phosphorus reserves. Upon this evidence Schaumann suggested that the antineuritic component was responsible, at least in part, for the mobilization of phosphorus. He was called upon to defend and to reaffirm his position upon the relationship of the antineuritic substance, "activator," to phosphorus metabolism (276, 278, 279).

Numerous attempts to separate the vitamin in pure form were made by Cooper, using various methods. He asserted that the active constituent of lentils was not precipitated by basic lead acetate, but was carried down almost entirely by ammonium molybdate (32). Similarly, in the Kossel-Kutscher fractionation procedure, Cooper found that the vitamin was only partially recipitated by silver nitrate, but was entirely deposited by silver nitrate-baryta. The residue obtained upon decomposition of the latter precipitate was dissolved in absolute alcohol, from which the active principle was thrown out by acetone. Crystallization from 50 per cent alcohol yielded an active white material, whose amount was insufficient for chemical examination (33). When the fractionation was repeated upon extracts from heart muscle, five grams of crude active material were obtained (36). Upon standing a few days in a desiccator the substance lost its curative action. Cooper concluded that the antineuritic principle was extremely labile.

In 1918 Abderhalden and Schaumann instituted an elaborate chemical investigation in their search for "eutonine," by which they designated the substance effective in attacks of alimentary distrophy and polyneuritis (3). By successively dissolving an active fraction in alcohol and precipitating by acetone, they obtained the "eutonine" from yeast in the form of a crystalline mass. This was active as a curative for polyneuritis in doses of 5 mgm., but it could not maintain body weight and life in pigeons fed polished rice. The "eutonine" was readily soluble in water and alcohol, but insoluble in ether, chloroform, and acetone. That it was not a pure compound was evident from an examination and analysis. Abderhalden and Schaumann believed that the active substance belonged in the betaine group.

Using rice meal as the starting material, Hofmeister was able to precipitate the antineuritic substance from an aqueous solution by potassium bismuth iodide. From the resulting purple red precipitate was obtained a crude crystalline mass which was active in doses of 5 to 10 mgm. (161). Addition of a gold chloride solution to the mass separated crystalline platelets and prisms, melting at 277°. The free base, "oridin," was prepared from this gold double salt; both products yielded a pyridine odor upon decomposition. With an empirical formula of $C_5H_{11}NO_2$, oridin is isomeric with betaine and valine; in its behavior Hofmeister maintained it was related to pyridine or piperidine compounds. The physiological significance of oridin is conjectural, since the activity of the crude mass was lost during its conversion into the free base. That Hofmeister contemplated a new approach to the isolation of the vitamin is evident from one of his posthumous papers (163). In it he discussed the conditions necessary for the adsorption of the vitamin. There he raised a question which has received scant attention; namely, the possibility that adsorption really plays a rôle in the precipitation of the vitamin by such substances as phosphotungstic acid, tannic acid, potassium bismuth iodide, and picric acid.

Myers and Voegtlin prepared a vitamin B concentrate from yeast by use of silver acetate-baryta and mercuric sulfate as precipitants (235). As the final product, they obtained spindle-formed crystals which possessed activity so long as they were surrounded by mother liquor; upon drying the material became inactive. They thought it probable that at least two basic substances were in the final solution, one of which was a histamine-like compound. By a similar method in which mercuric nitrate and ammoniacal silver nitrate were used, Marchlewski and Wievzchowski described the isolation of the vitamin in the form of

a picrate. The solution resulting from decomposition of the picrate had the same marked curative effect as the crystals (227, 228).

Ikeda submitted an alcoholic extract of rice bran to treatment with lead acetate, then with potassium bismuth iodide in alkaline medium. A syrup obtained in this way was separated into four fractions: purine bases, arginine, histidine, and choline-lysine. He found that the largest part of the antineuritic substance passed into the choline-lysine fraction, but upon further fractionation it appeared in the choline group. The active principle could not be isolated from there (171, 172). By an undivulged method Ganassini reported the separation of a highly potent powder from the hydrolytic products of yeast nucleic acid (132). In this connection it might be remarked that this highly complex acid was described by Drummond as having no growth-promoting property for rats (48).

Occasionally in recent years the use of phosphotungstic acid as a vitamin precipitant has been revived, although with doubtful success. Among the workers employing this acid has been Tsukiye, who adhered in the main to Funk's abandoned method (330). Tsukiye described the preparation of a gray-white powder, active in 5 to 6 mgm. doses. Three years ago Bowman and Yee gave an account of crystalline vitamin B, obtained by means of phosphotungstic acid (21). The pure crystals melted at 320°; they reacted with the Folin-Macallum uric acid reagent to form the characteristic blue color. In doses of 3 mgm. they cured polyneuritic pigeons.

In the experiments in which chemical precipitation has had a part, almost without exception, the yields of active material were so small that they made difficult any further purification, extensive animal tests, and chemical identification. This has been due to the disappearance of the antineuritic factor. Indeed it has been the experience of investigators that the loss of active substance was progressive during the numerous steps in long chemical procedures. Particularly was this true in methods involving precipitation by heavy metals; and it was not uncommon to reach the end of an investigation only to find that all activity had vanished. Perhaps this may partially be attributed to the persistent adsorption of the vitamin on the precipitates formed in removing the metals. But a more plausible view has come to light with the establishment of the multiple nature of vitamin B. No one can predict the number of substances which contribute to the composite action ascribed to vitamin B. If there were several factors, it is obvious that there would be only a limited number of procedures which would

keep them together so that the collective effect might become apparent. Of such methods few would be likely to yield any component in optimal amount.

Although the liberal use of metallic precipitants in the concentration of vitamin B is not highly regarded at the present time, Funk has not lost hope of finding a series of precipitating agents which will systematically carry such substances as vitamins from an unknown mixture into definitely recognized groups, as in qualitative analysis (130). He claims to have made progress in this direction by employing successively the various metallic salts of picric acid, picrolonic acid, and naphthol yellow S. The latter substance with silver picrate precipitates the greater part of vitamin B from a solution, according to him (129).

Feeding of known organic compounds. The administration of known chemical compounds to polyneuritic animals can by no stretch of the imagination be classified as a method of isolation of the vitamin. It excited expectation early, however, of shedding light on the chemical identity of the antineuritic substance. By testing synthetic substances which appeared likely to display some curative properties similar to the vitamin, the tedious process of applying doubtful methods of isolation would be spared. In 1909 Schaumann reported that nucleic acid from yeast exerted a temporary influence upon the paralysis in pigeons (271). Antineuritic activity was ascribed by Aron to another phosphorized organic substance, namely, phytin (10); but Schaumann (272), Fraser and Stanton (94), and Eijkman (72) could not concur with him in the finding.

Under the impression that the beriberi-vitamin probably possessed a pyrimidine structure, Funk fed a number of purine, pyrimidine, and pyridine derivatives to polyneuritic pigeons (108). Although this experiment was attended with more or less success in the case of allantoin, hydantoin, thymus nucleic acid, guanosine, adenosine and cytidine, no relationship could be established between the activity of these compounds and the identity of the vitamin. Williams and Saleeby agreed that allantoin was beneficial in certain cases of beriberi, but they added that it probably never effected a complete cure (353). Arginine, histidine, asparagine, lipoids of the lecithin group, and choline were excluded from the list of substances with antineuritic action by Chamberlain, Vedder, and Williams (26).

Cooper investigated the effect of several alkaloids upon paralyzed birds, and found that quinine and cinchonine displayed a temporary curative action (33). Strychnine prolonged the lives of the birds without relieving the cramps (32); cinchonidine was without any effect

(33). Extending his investigations into substances isolated from brain tissue, Cooper found that protagon, kephalin, cholesterol, and cerebrone were inactive (36). Abderhalden and Ewald asserted that administration of castor oil to polyneuritic pigeons resulted in prolongation of life (2).

Attaching significance to the association of nicotinic acid with the vitamin in curative fractions from several foodstuffs, Williams believed that the pyridine ring was the most promising nucleus for investigation (354). Upon testing the antineuritic properties of nicotinic acid and several of its derivatives, he found that the hydrochloride of the methyl ester of nicotinic acid exerted a marked but temporary improvement in pigeons; nicotinic acid, p-oxynicotinic acid, and trigonelline prolonged life, but brought little improvement in the paralysis. In the reaction of curative fractions with the Folin-Macallum reagent Williams thought that he saw a further clue to the chemical nature of vitamin B, in that it meant the presence of aromatic hydroxyl groups in the active substance (355). From tests upon a series of pyridine derivatives and related compounds, definite curative power was attributed to α-hydroxy-, 2,4, 6-trihydroxy-, and 2, 3, 4-trihydroxy-pyridines. Cinchomeric, quinolinic, 6-hydroxynicotinic, and citrazinic acids, α-hydroxypyridine, and tetrahydroxypyridine were without effect. All of the cures recorded, however, were with pigeons receiving their first treatment with the preparation. When the birds were treated with the same material a few days or weeks later, they invariably received no benefit. Apparently the substance had undergone some change, thereby losing its curative power. Since there was no evidence of decomposition, Williams inclined to attribute the change to isomerism.

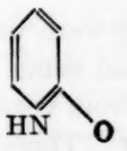
The dynamic form of isomerism, designated as tautomerism, was known to exist in hydroxypyridines; under governing conditions these substances could be made to react as enol or ketocompounds. Recognizing this fact, Williams prepared α-hydroxypyridine in two crystalline forms, needles and granules; and as a sodium salt. Each, he claimed, represented an isomeric form; each existed in equilibrium with at least one other. By treatment inducing molecular rearrangement any desired form could be made to predominate. Freshly crystallized needles were curative and retained some power for a week, at the end of which time they had passed over into the inactive granular form. The enolic sodium salt produced some relief in pigeons, but the results were questionable by reason of the presence of the non-enolic form in equi-

librium. β-hydroxypyridine, γ-hydroxypyridine, and γ-lutidone manifested curative properties, which were similarly lost with the lapse of time.

Since the inactive forms existed in equilibrium, the inability of the animal body to convert them into the desired curative form suggested to Williams that polyneuritis was not due to a deficiency of a substance *per se*, but to a want of potentiality for isomeric change. This lack of a certain type of potential energy could only be supplied by compounds possessing the power of isomeric change. By this theory the vitamin was not necessarily viewed as a hydroxypyridine; the natural vitamin occurring in foodstuffs was probably only one of several substances showing similar isomerism. Williams intimated that the isomerism existing in natural vitamin was responsible for its instability, which had prevented its isolation.

The foregoing hypothesis was strengthened when Williams and Seidell gathered results indicating that isomerism played a determinative rôle in the action of natural vitamins no less striking than in hydroxypyridines (356). From yeast they obtained a substance the activity of which was apparently not due to adhering mother liquor. After attempts had been made to purify it further, its antineuritic properties were lost and the resulting product was adenine. By suitable treatment, however, the adenine acquired antineuritic properties. They regarded the work of interest "not in its contribution toward establishing the composition or identity of this vitamine, which may or may not be a unique compound, but in affording corroboration of the theory [of isomerism]." Voegtlin and White (337), and Harden and Zilva (148) attempted to activate adenine by the method of Williams and Seidell, but the substance did not acquire antineuritic properties. They concluded that there was no direct relation between adenine and the antineuritic vitamin. Drummond reported that adenine would not support growth in the rat (48).

Having maintained that the antineuritic properties of hydroxypyridine depended upon an isomeric transformation, recognizable by two distinct crystalline forms, Williams sought to ascertain the chemical structure of the active modification. From results of animal experiments on several pyridine derivatives, he selected the structural feature which was associated in common with all the curative substances. He concluded that the active form of all hydroxypyridines possessed a structure best represented by pseudo-betaine (357, 358).



Pseudo-betaine

This view was strengthened by the practically complete cures produced by betaine. Judged solely from these curative tests, the inference might have been drawn that a betaine-like structure was an essential feature of the vitamin. Administration of betaine, methylpyridone, or trigonelline, however, failed to furnish protection for pigeons against the neuritic disorder. Williams did not regard this defection as a serious blow to his hypothesis, since he attributed the failure of the compounds in prophylaxis to the extreme lability of the active forms.

Even if antineuritic activity was shown beyond doubt to be due to the betaine ring, Williams reminded that it would not help greatly in establishing the identity of the natural "vitamines." The betaine configuration is theoretically possible in a large proportion of known biological substances; in fact, in all of the amino- and oxy-derivatives of cyclic nitrogen compounds. For this reason he could not believe that the "vitamines" belonged to a narrowly restricted group of compounds. Despite this elaborate theory of vitamin structure and activity, Williams' experimental evidence has not received confirmation. It is significant that Myers and Voegtlin found no antineuritic activity in stachydrine, trigonelline, allied betaines, and histidine esters (235). Harden and Zilva prepared the various forms of α-hydroxypyridine, but they stated that none were curative (148). To this Williams made a critical reply, but he conceded that the positive evidence of antineuritic action in hydroxypyridine was equally unconvincing, since the substance exerted no preventive effect (359). Using another series of synthetic organic compounds, Williams obtained a partial protection with trimethyluracil and 4-phenylisocytosine. Beyond this point he apparently abandoned all effort to adduce further evidence in support of his theory.

Another viewpoint of vitamin B structure was gathered from studies on its pharmacological action. Uhlmann declared that the antineuritic substance stimulated those glands innervated by the parasympathetic system. His vitamin preparations greatly increased the gastric, pancreatic, and biliary secretions, suggestive of secretin (332). On the other hand, the sweat glands reacted with marked secretion, and isolated hearts suffered paralytic effects, suggestive of pilocarpine (333). In

accord with the former findings were Voegtlin and Myers' observations that secretin solutions exhibited some antineuritic activity; and conversely a vitamin preparation from yeast stimulated the pancreatic and biliary secretions of dogs (340). These investigators asserted that the extract of the duodenum of a polyneuritic cat had no effect upon pancreatic flow, whereas the extract of a normal cat's duodenum causes an increased secretion. Further, secretin and vitamin B showed similar chemical and physical properties. They suggested that the two substances were identical. Judging from the effect upon blood cells, Downs and Eddy could not agree that a similarity existed between the substances (46). Likewise, Anrep and Drummond (9), and Cowgill (37) repeated the work of Voegtlin and Myers, and reached the conclusion that no identity existed between secretin and vitamin.

Cowgill cast further doubt upon Uhlmann's hypothesis by reporting that vitamin B preparations did not stimulate glands in a manner similar to pilocarpine (38). That the vitamin and pilocarpine have nothing in common has not been definitely settled, since Dutcher noted that this alkaloid produced definite relief in avian polyneuritis, although the response was not characteristic of vitamin preparations (53). Among other substances tested with some success by Dutcher were Kendall's thyroxin, desiccated thyroid, and tethelin. Accepting the then prevalent belief that thyroxin contained the indole nucleus, an assumption not supported by Harington's work, Dutcher extended his studies on antineuritic activity to other indole derivatives. He found that α-keto-β-propyl-indole and N-methyl- β-ethyl-indolinon possessed decided antineuritic properties (54). It is interesting that Seaman reported an extract of the thyroid gland curative for polyneuritic pigeons (285); Drummond found that this organ was without effect upon rats (49).

It will be recalled that Myers and Voegtlin observed the presence of a histamine-like substance in their vitamin fraction from yeast (235). Later, Danysz-Michel and Koskowski declared that histamine suppressed the paralytic symptoms appearing in rice-fed pigeons, but that it did not replace the vitamin. The action of this imidazole base was attributed to its stimulatory effect on the digestive glands (42, 193). Boyenval stated that it produced a similar effect on the rat (22). Abderhalden recorded success in curing the polyneuritic cramps by use of tyramine or hydroxyethylamine, as well as by histamine, but he attached greater significance to the failure of these substances as prophylactics (6), a view strongly supported by Peters (254).

From antineuritic action manifested by piperazine, dimethylpiperazine, and leucylpiperazine, Abderhalden has suggested that the vitamin may possibly owe its activity to the presence of a piperazine derivative (7). On the other hand, Funk, after returning to the study of pyridine derivatives, has asserted that nicotinic acid, its ethyl ester, and picolonic acid prevent loss of weight in rice-fed pigeons (230). Recently Iwata ascribed an antineuritic effect to phloroglucin (174). Kon administered glycine to pigeons without success (192). From this recital it should be recognized that the feeding of known organic substances has produced such variable results that it has disclosed little knowledge of vitamin structure. For the most part investigators have returned to the more arduous but assuredly more promising method of attempting to isolate the substance from its natural source.

Fractional precipitation. It is usually stated that absolute alcohol does not extract the antineuritic principle from natural foods; but that alcohol containing water is a solvent for the substance, and the higher the content of water, the better the solvent action of the alcohol. It would seem, then, that in instances where almost pure alcohol apparently extracted the vitamin the moisture contained in the working material, such as yeast and wheat embryo, had imparted a higher water content to the solvent mixture, and had thus increased its effectiveness in removing the active substance. If vitamin B is insoluble in strong alcohol, the principle should be precipitated from aqueous solutions upon addition of alcohol. The discordant results in this respect may be due to unappreciated conditions; perhaps the nature of the extract, and its relative freedom from inert material. It is possible that the apparent precipitation of the vitamin by alcohol is really the precipitation of inactive material upon which the antineuritic principle is adsorbed. Moreover, the solubility of a substance, such as the vitamin, varies with its relative state of purity. For these reasons it is to be expected that exact results obtained from the use of one extract would not be duplicated in work upon a different material.

In 1906 Eijkman stated that the antineuritic substance was not precipitated by alcohol from aqueous extracts of rice bran (71, 72). He concluded that the vitamin was soluble in strong alcohol. It is by no means certain, however, that Eijkman used sufficient alcohol in his precipitation experiments to warrant this inference. The same cannot be said of Fraser and Stanton. These investigators added increasing amounts of alcohol to an aqueous extract of rice polishings, until the

solution contained about 91 per cent alcohol by volume, without precipitating the active substance (94).

Despite this early recorded inability to throw down the antineuritic principle by alcohol, recent workers have achieved some success toward this end. Using an extract of rice bran that had been concentrated to a syrupy consistency, Murai precipitated the active constituents by addition of seven to ten volumes of absolute alcohol (234). The final product was a brownish-yellow, hygroscopic powder. In like manner Osborne and Mendel concentrated the growth-promoting vitamin from yeast (240). An aqueous extract was reduced to a pasty mass which was mixed with absolute alcohol and then evaporated. After repeated evaporations and frequent additions of alcohol, the resulting brittle mass was ground with absolute alcohol. The alcoholic solution was inactive for rats; whereas the residue, soluble in water but insoluble in alcohol, exerted a favorable effect.

Upon the basis of this finding Osborne and Wakeman formulated a method for preparing an extremely potent preparation of vitamin B (246). By fractionally precipitating an aqueous extract of yeast with alcohol they secured an active fraction in which most of the vitamin was present with only a small proportion of inert material. At the outset they prevented protein and much inactive water-soluble material from entering the yeast extract by boiling the cells with acidified water. Inasmuch as the extract was comparatively free from the products of autolysis, the conditions for fractionation were simplified.

A water extract from brewers' yeast concentrated to a small volume was poured into sufficient strong alcohol to make the spirit content of the mixture approximately 52 per cent by weight. The precipitate formed, known as fraction I, was inactive. Therefore, the filtrate was reduced in volume, and was poured into alcohol until the concentration of the solution became 79 per cent. The precipitate which appeared was designated fraction II. As before, the filtrate was added to sufficient alcohol to bring the concentration of the mixture to 90 per cent alcohol by weight. This precipitate was called fraction III. The filtrate from it was reduced to dryness and termed fraction IV. By feeding tests upon rats it was determined that most of the vitamin originally present in the yeast was concentrated in fraction II. Thus, Osborne and Wakeman were successful in concentrating the greater part of the vitamin in about 6 per cent of the dry yeast solids. That the active fraction was not free from mineral matter was shown by its

ash content of 10.65 per cent. This figure, however, was considerably lower than that recorded for any inactive fraction.

Levine, McCollum and Simmonds described the preparation of an active extract, to which fractional precipitation with ether might well have been applied (205). They were able to remove vitamin B from plant materials by means of glacial acetic acid. Since this solvent is readily miscible with ether, they concentrated their extract by throwing out a large quantity of inactive material with ether. However, no definite fractionation was attempted.

Dialysis. Sugiura conducted a unique experiment by placing a suspension of brewers' yeast in a collodion bag which was hung in the air for air dialysis, at room temperature, until the contents of the sac became dry (314). Five milligrams of colorless crystalline material were collected on the outer wall of the bag. These crystals were curative for polyneuritic pigeons.

Selective adsorption. After slowly filtering an extract of rice polishings through bone black, Chamberlain and Vedder found that the antineuritic substance was retained on the animal charcoal (25). The filtrate was inactive. Washing the charcoal with water appeared to remove some of the vitamin. In later experiments they extracted the active bone black with ether, absolute alcohol, and water, but none of these solvents removed the antineuritic substance (26). They attributed the failure of water in this case to the use of a purer charcoal, which apparently possessed greater adsorptive power. Cooper was unable to altogether confirm the adsorption of vitamin by bone black, since an active solution filtered six times through animal charcoal was still curative for polyneuritic pigeons, although its activity had apparently been reduced 30 per cent (33).

Besides bone black, several substances have been shown to have an adsorptive affinity for the antineuritic vitamin. Harden and Zilva stated that the active principle was removed from autolyzed yeast by dialyzed iron, and that it was recovered from the precipitate by extraction with 95 per cent alcohol (149). Myers and Voegtlin found that olive oil and oleic acid also separated the vitamin from yeast filtrate (235). In this instance 0.1 per cent hydrochloric acid was used in extracting the active principle from the adsorbent. In the same communication these investigators expressed their disfavor for mastic and ferric phosphate as adsorbents for the vitamin, due to their lack of specificity. Only a portion of the vitamin content of a rice extract was adsorbed by infusorial earth in the proportion used, according to Brill

(23). It was the experience of Emmett and McKim that the kieselguhrs, including infusorial earth, "Celite B," and "filtercel," did not adsorb the antineuritic substance from yeast extracts (77).

In 1916 Seidell announced that vitamin B was adsorbed from autolyzed yeast by a particular variety of fullers' earth known as Lloyd's reagent (286). This vitamin-Lloyd's reagent combination was termed "activated solid" by him. Subsequently, Emmett and McKim stated that the ordinary form of fullers' earth adsorbed the vitamin as readily from yeast as did Lloyd's reagent (77); but Myers and Voegtlin did not regard the earths as satisfactory reagents because of their lack of specificity (235). Concerning the action of the silicate adsorbents upon the growth-inducing vitamin, Eddy asserted that Lloyd's reagent removed the active substance from pancreas extracts (58); but Funk complained that a complete separation of the vitamin from solution could not be effected by this adsorbent (118). With slight difference in detail Seidell's finding of the vitamin-fullers' earth complex has been abundantly attested.

Within a short time Seidell and Williams learned that the antineuritic substance could be removed from the activated fullers' earth by extraction with sodium hydroxide or baryta solution (356). By further purifying the material yielded by this adsorption method, Seidell secured a relatively stable silver-vitamin compound (288). In his process the antineuritic substance was removed from the activated solid by baryta solution, and the active extract was clarified by lead acetate precipitation. The filtrate, freed from excess lead, was concentrated to a small volume under reduced pressure. Several crops of white, amorphous material separated, but all were inactive. The solution finally became a thick, viscous mass. When silver nitrate solution was added to this residue dissolved in water, a voluminous precipitate formed which was inactive. Addition of ammoniacal silver nitrate to the filtrate produced another precipitate, which contained a readily crystallizable inactive substance and the difficultly crystallizable vitamin. The filtrate therefrom was practically free from the antineuritic principle. After removal of the inactive crystalline compound from the precipitate, the vitamin was again thrown down as a silver compound with its antineuritic property unimpaired.

An examination showed that the insoluble compound formed by silver nitrate precipitation was mainly adenine; while the inactive crystalline substance produced by ammoniacal silver nitrate precipitation was a base closely related to histidine, although it was neither

histidine, histamine, nor carnosine (289). By this time Osborne and Wakeman's method of fractional precipitation by alcohol had appeared in the literature; therefore, Seidell used this active fraction as the starting point for adsorption of the vitamin by fullers' earth (290). In this way the presence of adenine, one of the interfering substances simultaneously adsorbed with the vitamin, was avoided.

After incorporation of this modification into his method, Seidell determined that approximately one-third of the solids of the extract from fullers' earth was precipitated as silver compounds, containing somewhat more than half of the antineuritic principle (291). He attributed the incomplete precipitation of the vitamin to the considerable solubility of its silver compound. Nitrogen determinations used as criteria of purity led him to believe that the vitamin fractions were mixtures of two or more analogous bases. The chemical similarity of such compounds was inferred from their common adsorbability on fullers' earth, their common extraction by aqueous alkali, and their common precipitation by ammoniacal silver nitrate. Seidell concluded that the antineuritic substance could not be advantageously separated from the other bases by silver precipitation.

While working with Bertrand in Paris, Seidell showed that somewhat more than 80 per cent of the vitamin present in an extract from activated solid could be converted into a crude picrate (15). Fractionation of this product by recrystallization from 95 per cent acetone yielded two crystalline picrates (293). The one, obtained from the acetone-insoluble portion of the crude picrate, consisted of pale yellow rods or prismatic crystals, melting at 202°. These were inactive. The other, derived from the acetone-soluble portion of the product, yielded aggregates of irregular-shaped crystalline flakes. In doses as low as 2 mgm. daily these crystals protected rice-fed pigeons from loss of weight. Combustion analyses of the two picrates gave results indicating an empirical formula of $C_6H_{18}O_2N_3OHC_6H_2(NO_2)_3$. Seidell suggested that the two compounds were very closely related; indeed that they might be tautomeric forms of the same compound. Later he remarked that the results from combustion analyses on the active picrate had not been sufficiently satisfactory for the calculation of a formula (294). At the same time he indicated that a portion of the nitrogen of the potent compound was present in the amino form. Finally Seidell was able to remove the picric acid from the vitamin picrate by means of nitron. The resulting free base retained its antineuritic properties (295). The molecular weight of the base, determined by freezing point method, was

201; a combustion analysis gave results corresponding to the formula $C_8H_{15}O_3N_3$. One amino nitrogen group was present in the molecule.

In contrast to this intricate and almost microchemical procedure, Seidell has turned his attention to the development of a somewhat less complicated and large scale production of the vitamin in a stable form (296). "The several steps of the method are: removal of the yeast protein by boiling; adsorbing the vitamin from the resulting solution by means of fullers' earth; extracting the activated solid with sodium hydroxide; acidifying and concentrating by vacuum distillation; removing the sodium as $Na_2SO_4 \cdot 10H_2O$ and the non-active organic material by means of methyl alcohol; finally, converting the active viscous residue to a non-hygroscopic dry powder by treatment with strong ethyl alcohol."

Kinugasa and Hattori used acid clay for the adsorption of vitamin B from solutions of yeast autolysate or rice bran (189). The antineuritic substance was removed from the clay by baryta. The resulting solution, freed from barium, was fractionally precipitated with silver nitrate and silver nitrate-baryta. When the precipitate produced by the latter reagent was decomposed, it yielded an active picrate, melting at 195–222°. They stated that the crystalline compound was still impure. The active substance regenerated from the picrate gave only a weak diazo reaction, but a deep blue coloration with the Folin-Macallum reagent.

Using the Osborne-Wakeman active fraction as a starting material, Levene and van der Hoeven further concentrated its antineuritic content by precipitation with barium hydroxide. Although the product obtained by this step was from five to ten times as potent as the initial fraction, the procedure was attended with a loss of about half of the vitamin originally present (200). They turned then to the use of various adsorbents, by which means they prepared extraordinarily active concentrates (201). From the behavior of Lloyd's reagent toward the vitamin they formulated a theory concerning the adsorbing mechanism of the former substance. The surface of the reagent acted as a salt of a weak acid, they said; and it was capable of a double exchange reaction with slight complications. They assumed from this fact that "its adsorbing power would be affected by the degree of ionization of the active surface of the adsorbent, and the degree of dissociation of the substance to be adsorbed." Since both conditions depended upon the hydrogen ion concentration of the medium, Levene and van der Hoeven postulated the existence of a certain pH for each substance

when its adsorption was optimal. This theory was confirmed by their finding that the optimal adsorption of the vitamin upon Lloyd's reagent occurred at pH 4. The optimal extraction of the active principle took place at pH 9.

Extending their studies, they found that silica gel would adsorb the active material most effectively at pH 5. What was striking in this instance was the discovery that the vitamin could be removed from the gel not only by alkalies but also by acids. However, only a part of the antineuritic substance was extracted at pH 3; the larger portion was removed at pH 9. When material obtained by barium precipitation was submitted to this silica gel process, its activity was increased 200 to 400 times. At this point Levene and van der Hoeven introduced several new steps into their procedure, and rearranged their plan for concentrating the vitamin.

Under the revised process the antineuritic principle in the Osborne-Wakeman fraction of yeast was precipitated successively by basic lead acetate, and by barium hydroxide (202). The active material yielded by this treatment was adsorbed upon silica gel, and then was removed under the prescribed conditions. The final product contained 4.6 per cent phosphorus and 15 per cent nitrogen. It showed positive reactions for carbohydrates, and was active upon rats in about 0.1 mgm. substance. Levene and van der Hoeven pointed out that the co-adsorption or even introduction of mineral impurities, particularly phosphates, was a very disturbing factor in the purification of the vitamin.

When the active material obtained from lead-barium treatment was precipitated with absolute alcohol in a strongly acid medium, the troublesome inorganic salts were removed. Such a purified preparation furnished after silica adsorption a final product free from phosphorus. It contained only 4.2 per cent nitrogen. However, its activity of 0.6 mgm. in a daily dose was much less than that of the phosphorized product. Apparently purification of the material by removal of inorganic salts was attained with considerable loss of activity, and the resulting product was not so effectively improved by silica adsorption as was the phosphorized material. Levene and van der Hoeven remarked that the phosphorus-containing impurity seemed essential for selective adsorption by silica.

Although the preparation derived from the foregoing method was extremely potent, it was a complex mixture. In addition, its further purification led to considerable loss in activity. Therefore, Levene and van der Hoeven abandoned this process, and developed a more economi-

cal method of concentration. Adjustment of a solution of the Osborne-Wakeman fraction to pH 4 formed an inactive precipitate (203). The filtrate was deaminized by nitrous acid, and then was submitted to silica gel adsorption. The final product, removed from the adsorbent, was a complex mixture comprising carbohydrates, nitrogenous substances, and esters of phosphoric acid (204). It possessed no amino groups, but it contained sulfur in the thiol form. Levene and his associate remarked that the sulfur content had increased with purification, but they deemed it would be premature to assert whether the sulfur was a part of the active substance. An analysis of the final product gave the figures (calculated ash free): C = 41 per cent; H = 6.7.; N = 9.0; P = 16.5; total S = 3.9, thiol S = 2.0; and NH_2 = 0. This preparation was active for rats in daily doses of 0.08 mgm.

Whereas Seidell had essayed to isolate the substance preventing polyneuritis and loss of weight in the pigeon, and Levene and van der Hoeven the vitamin inducing growth in the rat, Peters sought to separate the curative principle relieving head retraction in the pigeon. Peters termed this substance torulin, and stated that it might or might not be the same vitamin with which Seidell, and Levene and van der Hoeven had dealt. In Peters' concentration of torulin, an aqueous extract of yeast was clarified by successive treatments with lead acetate, and with acid mercuric sulfate (254). The active substance in the filtrate was adsorbed upon norite, a birchwood charcoal; it was then removed from the adsorbent with 50 per cent alcohol containing hydrochloric acid. This extract was active in daily doses of 3 mgm. solid.

Later Peters with Kinnersley extended the procedure of purifying torulin by introducing subsequent steps which involved precipitation of inert material by lead acetate, by dialyzed iron, and by ethyl alcohol; fractionation in an alcohol-ether mixture; and finally fractionation by silver salts (186). They obtained a yeast concentrate which was active in daily doses of 0.084 mgm. When this method was applied to large scale production, complications arose in the pre-charcoal steps (187). Accordingly, a treatment with baryta for removal of gums was inserted between the lead acetate and acid mercuric sulfate precipitations. Then the torulin was adsorbed upon norite at pH 7. Kinnersley and Peters stated that the curative substance was best removed from the charcoal adsorbent by two extractions: one with 0.1 normal hydrochloric acid at a pH acid to congo red, the other with acid 50 per cent alcohol. The combined extracts, when subjected to alcoholic fractionation, yielded

a product containing 15 to 25 per cent nitrogen and showing activity in a daily dose of 0.15 to 0.30 mgm.

During the past year Jansen and Donath, working in the same laboratory in which Eijkman made his fundamental discovery, announced the isolation of the antiberiberi vitamin in crystalline form. In their method the antineuritic substance in rice polishings extract was adsorbed at pH 5 upon acid clay, from which it was subsequently removed by baryta. When the clay extract was subjected to fractionation with silver nitrate-baryta, more than 50 per cent of the vitamin was precipitated by these reagents at pH 6.5. The decomposed precipitate yielded a solution which was decolorized by norite without loss of the vitamin (177). This latter step is interesting in the light of Peters' use of the charcoal as an adsorbent for the antineuritic substance. The clear filtrate was treated with phosphotungstic acid; the resulting precipitate was decomposed; and its solution was concentrated to dryness, and taken up in absolute alcohol.

Addition of platinic chloride precipitated practically all the vitamin and only one-fourth of the solid matter present in the alcoholic solution. The solution obtained from decomposition of the platinum precipitate contained one-fourth of the vitamin originally present in the polishings, and at least two other substances associated with the active principle (178). That this number of substances was present in the admixture became evident from an acetone-alcohol treatment, which yielded three fractions: the first was insoluble in absolute alcohol; the second was soluble in absolute alcohol, but insoluble in acetone (this contained the vitamin); the third was soluble in both absolute alcohol and acetone. By further purification of the second fraction the vitamin was obtained in crystalline form as a hydrochloride. An alternative method involved treatment of the second fraction with picrolonic acid, whereby the vitamin separated as the insoluble picrolonate. From this compound could be formed the hydrochloride.

The pure vitamin hydrochloride consisted of rosettes of small bars, melting at 250°, according to Jansen and Donath. The compound was readily soluble in water and methyl alcohol, but less readily in ethyl alcohol. With gold chloride the substance yielded a double salt, the analysis of which gave figures corresponding to formula $C_6H_{10}ON_2$ for the free base. Since the vitamin responded with a positive Pauly's diazo reaction, Jansen and Donath stated that the vitamin probably contained an imidazole or a pyrimidine ring. The yield by their method was 100 mgm. vitamin hydrochloride from 3 kgm. rice. What was probably the

most important feature was the test for activity of the compound upon bondols (native rice birds). Jansen and Donath reported that 0.003 mgm. vitamin hydrochloride administered daily to a bondol was sufficient to maintain health for three weeks in birds which otherwise would have contracted polyneuritis.

The effectiveness of Jansen and Donath's vitamin hydrochloride in both curing and preventing polyneuritis was attested by Eijkman (76). He stated that a 0.5 mgm. dose of the compound brought rapid recovery in chickens, and a 0.2 mgm. dose produced the same beneficial result in pigeons. Further, the addition of as little as 4 mgm. vitamin hydrochloride to a kilogram of polished rice ration prevented the appearance of polyneuritis in pigeons. Eijkman reported that the active crystals gave a positive test with the Folin-Macallum uric acid reagent.

In contrast to these evidences supporting the isolation of the vitamin, it must be mentioned that Kinnersley and Peters applied the Jansen and Donath method to some yeast fractions without success (187). Furthermore, the substance producing the Pauly reaction did not fractionate with the substance possessing antineuritic activity. Kinnersley and Peters hesitated to cast doubt upon Jansen and Donath's work; rather the English investigators explained the failure of the method in their hands by assumption that the yeast used by them might have some peculiarity not possessed by rice polishings used by Jansen and Donath. It was also suggested that torulin might possibly be distinct from the antiberiberi vitamin.

MULTIPLE NATURE OF VITAMIN B. In a matter open to two interpretations, each of which is in diametric opposition to the other, it is natural that the simpler notion should prove the more acceptable. Particularly is this true where neither view has the advantage in evidence; where the one side falls short in proof, but the other is just as far from establishment. Such a situation, however, is likely to bring a shifting of views with the lapse of time. The facts available at the moment are doubtless so meager that they lead inevitably to imperfect vision which is corrected only when subsequent increments of knowledge cast their light. In no other instance are these circumstances better typified than in the contrary views concerning the nature of vitamin B.

During the decade which extended to 1926, most biochemists interested in vitamin B assumed, either avowedly or tacitly, that the antineuritic principle, discovered by Eijkman, and the water-soluble growth-inducing factor, discovered by McCollum and Davis, were identical. This view was accepted upon evidence that sources of the antineuritic

vitamin effectively promoted growth in the rat, and that sources of the water-soluble growth factor contained the specific curative for polyneuritis in pigeons. This parallelism of antineuritic power and of growth production in the same material became more striking when rats deprived of the water-soluble growth vitamin developed polyneuritic symptoms identical with those in pigeons denied the antineuritic principle. Furthermore, similarity in solubility relationships and chemical behavior tended to substantiate the idea that these two factors were actually one and the same substance, vitamin B. This was the simplest conclusion.

It must not be imagined, however, that the possibility of the antineuritic principle being distinct from the growth factor was definitely dismissed from mind. From the earliest work upon the chemistry of the antineuritic vitamin, investigators had been groping with the thought that this substance was multiple in nature, and had been relying upon this interpretation to explain occasional data that did not coincide with well-established facts. It was not to be expected that the idea that vitamin B was one substance, possessing antineuritic and growth properties alike, would be unanimously adopted. But for a long time there was insufficient evidence either to definitely discredit this theory of identity or to establish the existence of separate substances. True there were minor discrepancies in the occurrence and in the behavior of the factors, but the evidence at best was only highly suggestive, so the theory of one substance held its ground.

Eventually a growing conviction that vitamin B was multiple in nature found firm support. It has been established recently that the vitamin is not one substance, but at least two; how many more, no one can safely predict. Curiously this development has its counterpart in the field of endocrinology. Just as vitamin B was once regarded as one principle, which manifested two distinct physiological properties, growth and prevention of polyneuritis; but is now accepted as comprising two substances, each of which has a specific property: so pituitrin was viewed as one substance, which possessed three pharmacological properties, pressor, oxytocic, and diuretic-antidiuretic effects (8); but is now described in some quarters as constituting two principles, one responsible for the pressor and the diuretic-antidiuretic effects, the other for the oxytocic activity (180).

Early views. After the isolation of several substances from the vitamin fractions of yeast and of rice polishings, Funk declared that at least two antineuritic compounds were necessary for effecting a cure in

polyneuritic pigeons (109). Essentially the same conclusion, with however quite another interpretation, was reached by Vedder and Clark in their suggestion that two vitamins were necessary for normal metabolism: the one preventing neuritis, the other preventing cardiac failure, edema and general prostration (335). According to their view, wet beriberi and dry beriberi were two distinct conditions, each being caused by a deficiency of a separate vitamin; a third type of the disease, characterized by mixed wet and dry symptoms, resulted from deprivation of both vitamins. McCarrison, as is not uninteresting to note, entertained almost the same idea, that two factors were concerned in the prevention of avian beriberi: an antineuritic substance, and an anti-edema substance (216).

Apparent confirmation of this theory was seen in Vedder and Williams' report that administration of unhydrolyzed extract of rice polishings to adults in cases of wet beriberi or acute cardiac insufficiency brought prompt dissipation of edema and relieved the condition of the heart, but that administration of the same material in cases of dry beriberi caused no improvement in paralytic symptoms (336). Furthermore, they stated that Funk's antineuritic vitamin cured the paralysis of dry beriberi, but that the filtrate from the phosphotungstate of Funk's base, supposedly free from the vitamin, induced rapid recovery from wet beriberi. Criticising the supposition of specific vitamins for two types of the disease, Schaumann pointed out the seeming contradiction that unhydrolyzed extract of rice polishings should be inactive for paralysis in dry beriberi, whereas Funk's vitamin, isolated from the extract after hydrolysis, proved effective for that condition (280).

From Schaumann's criticism it must not be supposed that he attached no weight to the possible multiple nature of the vitamin. He had already commented upon his success with rice bran and with yeast, not only in relieving paralysis in animals but also in increasing their weight; on the other hand, extracts of these foods often failed in one or the other respect. For this reason Schaumann attributed the vitamin properties to several substances, acting collectively and mutually supplementing each other. Cooper received a similar impression after he had noted the lack of correspondence between antineuritic and weight-maintaining properties in certain foodstuffs (32). This inconsonance, he said, indicated that the substances responsible for the properties were not identical. Much this feeling doubtless prompted the criticism of Funk's crude vitamin product, which induced rapid recovery in polyneuritic pigeons, but which could not maintain the birds on a polished rice diet

for more than a few days (109). To meet this exception to his results, Funk prepared a vitamin fraction, the action of which was complete in that it possessed at the same time antineuritic and weight-maintaining properties; as a consequence he denied the necessity of assuming the existence of several vitamins (111).

Another slant upon the importance of unidentified dietary substances was presented when Hopkins found that rats fed purified food mixtures failed, unless milk was added to the ration (165). He was cognizant that a deficiency in certain "accessory foodstuffs" would induce a specific disease; he thought it was equally as certain that a similar deficiency might result in failure of growth. Commenting upon Hopkins' success with the addendum of milk to a purified regimen, Funk reiterated the view that deficiency diseases "can be prevented and cured by the addition of certain preventive substances, ... vitamines," although he actually had evidence for the existence of only one. Further, "I suppose that the substance facilitating growth found in milk is similar, if not identical, with the vitamines described by me" (105).

Within a short time McCollum and Davis declared that a small quantity of substance found in butter fat or in the ether extract of egg-yolk was necessary to supplement a diet of purified protein, starch, and salts for growth in the rat (217a). Apropos of this finding Funk asserted, "There is . . . . probability that the growth-promoting substance belongs to the group of vitamines in the broad sense, and I would like to call it the growth vitamine." He tried to effect a correlation of his vitamin, which protected against beriberi, with the newly discovered fat-soluble substance, which was necessary for growth in rats. But when his beriberi-vitamin was incorporated in a purified diet, cessation of growth in rats resulted (114). From this sequel he concluded that the "growth vitamin" was not identical with his antineuritic vitamin. Although this conclusion later proved correct, its truth was not convincingly demonstrated by his evidence. His diet was too inadequate in certain respects for the promotion of growth.

The reason for Funk's failure became clear when McCollum and Davis showed that aside from the unidentified substance carried by butter fat another factor, which was soluble in water, was necessary for growth in the rat (217). Their theory of an adequate diet was summarized in the statement that "certain at present unidentified substances aside from protein, carbohydrates, fats and salts are indispensable for growth or prolonged maintenance, and furthermore that there is a class of such accessories soluble in fats and another soluble in water and alcohol"

(218). These two factors were not the same; each was necessary for growth in the rat; neither could supplant the other. It was now apparent that Funk had been trying to replace the fat-soluble substance with his water-soluble antineuritic vitamin. If he had added butter fat along with his beriberi-vitamin in the ration, he might have secured growth and elucidated the problem of a complete diet.

Assuming now, as previously, that the vitamins were associated in some manner with McCollum and Davis' dietary principles, Funk and Macallum approached the problem from another direction, namely, by testing foodstuffs for the presence of factors preventing polyneuritis and promoting growth (116). When they added butter fat to a purified diet, rats failed in growth and pigeons contracted polyneuritis. Funk and Macallum therefore concluded that the beriberi-vitamin was not in butter fat. When they employed diets containing both butter fat and yeast, they obtained growth in the animals. They then declared that "the growth-promoting factor is beyond question in yeast." This statement was true in part, but it carried the implication that only one factor, the beriberi-vitamin, was needed for growth. Actually their success with yeast in the diet hinged upon the inclusion of butter fat with it, since McCollum and Davis had already advised that two types of substances were required for successful nutrition. The recital of these events is interesting merely as an illustration of the confusion that reigned during this period.

It is apparent that the trend of investigation at this time was toward showing the relationship between Funk's vitamins, which were preventives for specific diseases, and the unidentified substances necessary for growth, the existence of which had been established by Pekelharing, Hopkins, and McCollum and Davis. Their identity was foreshadowed when Funk reported that the so-called synthetic diet, consisting of casein, fat, starch, sugar and salts upon which rats invariably failed to thrive, produced polyneuritis in pigeons (115). That the diet which was lacking in the growth-promoting substances was likewise essentially free from Funk's antineuritic vitamin became at once evident.

The final step in demonstrating the relation of the antineuritic vitamin to the two classes of growth substances, the one soluble in fats, the other in water, was taken by McCollum and Kennedy (220). Finding that pigeons could be brought into a polyneuritic state by feeding a diet free from both substances essential to the rat, namely, fat-soluble A and water-soluble B, and that they could be cured by addition of water-soluble B alone, these investigators attributed the polyneuritis in birds

to the lack of the specific principle, water-soluble B. The logical conclusion was that Funk's antineuritic vitamin and the growth-promoting water-soluble B were the same substance. Likewise the nutritive requirements of the two species, represented by the pigeon and the rat, were now clearly understood. The pigeon needed only water-soluble B for the prevention of polyneuritis, but the rat required fat-soluble A in addition to the water-soluble vitamin for growth and well-being.

But this latter fact was not yet fully appreciated by Funk. Using Lloyd's reagent for adsorbing the growth-promoting substance from yeast, according to Seidell's method, Funk added the activated solid to a purified diet without a source of vitamin A (118). That he was able to obtain growth in rats upon this ration plus the activated solid, but without vitamin A, may be attributed to the shortness of his test period. His omission of butter fat from the diet shows his persistent failure to realize the need of two distinct vitamins for the rat; indeed his search for *the* growth-promoting substance in yeast accentuates his mistaken notion concerning the number of essential substances for growth and their relation to his antineuritic vitamin.

In formulating his "vitamine" hypothesis, Funk had stated that many other diseases besides beriberi, namely, scurvy, pellagra, rickets, and sprue, were due to deficiency of specific vitamins (117). McCollum and Pitz recognized the possibility that extracts which furnished the water-soluble B, capable of promoting growth in rats and inducing cure of polyneuritis in pigeons, might contain the entire list of Funk's protective substances (222). From the evidence at their disposal they concluded "that the water-soluble B contains but a single substance which is physiologically indispensable, rather than a series of such substances."

The earlier assertion that water-soluble B was identical with the antineuritic vitamin was not without ample confirmation. For the elucidation of the nature of the growth-promoting substance, Funk and Macallum applied the method which had proved more or less fruitful in the chemical investigation of the beriberi-vitamin (119, 226). Such a precipitant as phosphotungstic acid, which had been used in the separation of the antineuritic vitamin from yeast, was now employed in studies upon water-soluble B. In every case the fractions which enabled animals to grow, *i.e.*, contained the water-soluble principle, were those in which Funk had previously found the antineuritic vitamin. Furthermore, the two principles showed identical behavior toward solvents, and toward the action of acids and alkalies. Under such circumstances

Funk and Macallum essentially agreed with McCollum and Kennedy that the growth-promoting substance was identical with the beriberi-vitamin. And from the results of a similar investigation Drummond was led to the same conclusion (48).

Studies on the solubility of water-soluble B in organic solvents gave McCollum and Simmonds occasion to reiterate their stand that the water-soluble growth and anti-neuritic vitamins were the same substance (223). They said: "The probability that there should be two or more physiologically indispensable substances in what we term water-soluble B, both or all of which should show the same solubility relations with three solvents [alcohol, acetone, and benzene], is relatively small and lends support to our view that the substance which protects animals against polyneuritis is the only essential complex in the extracts described." This theory made polyneuritis in the rat, loss in weight in the rat on an otherwise complete diet, and polyneuritis in the pigeon referable to one vitamin.

In this way the terms water-soluble B and antineuritic vitamin came to be used interchangeably by many, until eventually the designation vitamin B was applied to both. This view is reflected as late as 1924 in the *Report on the Present State of Knowledge of Accessory Food Factors* (*Vitamins*) by the British Medical Research Council Committee (229). In this monograph is stated: "It is now very widely believed that the water-soluble B and antineuritic factors are identical. Although this identity is generally accepted, it cannot be said to be absolutely established. A number of apparent discrepancies in distribution and properties (solubility, stability towards heat) have been recorded, but the methods so far employed have not been sufficiently accurate to demonstrate either definite difference or absolute identity. . . . . In this report they will be provisionally regarded as identical and termed vitamin B."

Somewhat dubious and hesitant about accepting the idea, Osborne and Mendel asserted, "Whether or not the antineuritic component is identical with the growth-promoting one is a question which as yet has received no definite answer" (240). Again they stated, "That the substance which induces the remarkable recoveries which have been described in these cases [polyneuritis] is identical with the water-soluble hormone which is so essential for growth and maintenance is as yet merely a matter of conjecture" (241). But they offered no facts which would contradict the wealth of evidence pointing to such an identity.

Meanwhile, however, investigators interested in the isolation of the

beriberi-vitamin had obtained data which intimated that its antineuritic effect was due to the composite action of several substances. In this regard it must not be forgotten that R. R. Williams believed that polyneuritis was not due to a deficiency of one substance *per se*, but to a lack of a certain type of potential energy which might be supplied by any substance possessing the power of a particular isomeric change (355). Amplification of this view came from Williams and Seidell's finding that activated fullers' earth after alkali treatment, although absolutely preventing the development of polyneuritic symptoms in polished rice-fed birds, failed to protect them against loss of weight (356). "Therefore," wrote these investigators, "it appears that the complete physiological activity of the constituents of autolyzed yeast adsorbed by fullers' earth is a combination of antineuritic and weight-maintaining functions, and it is the latter only which is materially affected by alkalies."

Upon different evidence Emmett and McKim essentially embraced the foregoing view. They stated that activated fullers' earth, when administered as a supplement to polished-rice, occasioned some increase in weight as well as recovery in polyneuritic pigeons, but that it did not produce a gain in weight to an extent characteristic of unhulled rice (77). Apparently the activated solid was not a complete addendum to the polished-rice diet; therefore, they inferred that two vitamins were present in the unhulled rice grain, one of which cured polyneuritis and the other of which governed increase in weight. Of the two principles the fullers' earth preparation contained principally the curative one with only a small percentage of the other.

Although their finding did not concur with that of Williams and Seidell in all respects, Abderhalden and Schaumann similarly attested that alkali treatment could create a distinction between the antineuritic and the growth-promoting principles (3). As has been noted, they chose the name "eutonine" for the organic bases which induce recovery in alimentary distrophy, of which the nervous disorder, polyneuritis, was regarded as only one aspect. As a collective name for all the unidentified dietary constituents which limit the biological value of a diet, they proposed the designation "nutramine," equivalent to the general term "accessory foodstuffs." According to these investigators, polished rice could be supplemented satisfactorily for pigeons by eutonine together with another product prepared from yeast by sodium hydroxide treatment, although neither preparation alone was an effec-

tive addendum. Hence, they concluded that an alkali resistant factor was present in the yeast product.

That the water-soluble substance was multiple in nature received further support from the contrasting effects of foodstuffs and of extracts from them. As had been known for some time, a small amount of yeast so supplemented polished rice that the combination maintained weight and prevented polyneuritis in pigeons. Alcohol or acetone extracts of the yeast similarly added, however, could not bring about maintenance, although they possessed pronounced antineuritic activity. Therefore, Abderhalden drew the conclusion that the feeding of polished rice led to the appearance of numerous deficiency symptoms which were conditioned by the lack of several substances in the food (4). Moreover, the favorable effect of yeast upon alimentary distrophy was in all probability, he said, not attributable to one compound, but to several compounds with different effects. He then intimated that one or several of the nutramines had been removed from the yeast during alcoholic extraction; on the other hand several had remained untouched. Under such circumstances the extract would not show the full effect of the food, as was seen, since the collective action of all the nutramines present in proper proportion would probably be necessary. Reasoning in this manner that the yeast cell must contain many different active components, Abderhalden postulated the existence of such a series comprising "Atmungstoffe," growth substances, and maintenance substances (6). However these theoretical considerations extend so far beyond any firm basis that further discussion becomes unprofitable.

In a similar strain Hofmeister broached the thought that antineuritin might not correspond to vitamin B *in toto* (162). He reminded that the pigeon test for antineuritin depended upon the cure or prevention of polyneuritis; that the rat test depended upon growth. Granted that yeast and rice bran extracts not only cure polyneuritis in birds but also bring an increase in weight and recovery in rats, Hofmeister reasoned that the growth effect of the extracts might not be due to the antineuritin but to an accompanying substance, which was an independent metabolic agent.

The situation as it existed in 1919 was portrayed in some detail by Mitchell (233). He pointed out that belief in the identity of the antineuritic vitamin and water-soluble B rested upon the following grounds:

> (1) The distribution of the two substances in natural food products is very similar and the correlation between the actual amounts . . . . found in different products appears to be close. (2) The lack of known sources of water-soluble B

in the diet of various species of experimental animals seems generally, if not always, to result in symptoms of nerve degeneration and paralysis. (3) Extracts of natural food products possessing growth-properties are said to contain very probably only one indispensable vitamine, though supplementing satisfactorily a ration containing no other possible source of antineuritic vitamine. (4) The solubilities of the two vitamines in the common solvents are said to be identical. (5) Attempts at isolating the two vitamines have shown that they possess identical precipitants and adsorbents. (6) The stabilities of the two substances, especially to acids, alkalies, and elevated temperatures, seem to be similar if not identical.

Assembling and analyzing the existing data on each point, Mitchell cast doubt upon the identity of the two substances, but his interpretation in many instances was not impressive. It could not have been otherwise. The evidence which could be mustered was too incomplete to lend more than slender support to the distinction between the two substances. On the other hand, that any contradictory data could be marshalled into an argument against the identity of the vitamins was not surprising; it is not unusual that well-established facts fail confirmation in some hands, to say nothing of evidence in an issue, such as this, which was not definitely settled. Taken by and large, Mitchell's review was highly suggestive, but it contributed no additional facts to the subject. In this connection it is pertinent to note that he outlined a crucial experiment which, he predicted, would establish the truth concerning the third point, but so far as is known he has never recorded its outcome. In short, his argument, lacking as it did any new element, furnished no convincing reason for believing that the two vitamins were independent substances, but it did focus much-needed attention upon this possibility.

Much of the data transcribed by Mitchell has already been recorded in the present paper in the sections on solubility, stability, and chemical isolation, and the existing discrepancies were then indicated. It might not be amiss to mention a part of the evidence dealing with Mitchell's first point, namely, the distribution of the substances, which has not received previous treatment. According to Voegtlin and his associates, all of the antineuritic vitamin is contained in the embryo of the wheat kernel (339, 341, 342); but Osborne and Mendel, and Bell and Mendel state that water-soluble B is distributed throughout the wheat kernel (243, 14). The embryo induced recovery in polyneuritic pigeons (342); on the other hand it provided only maintenance and did not support growth in the mammal (243, 30), unless fed in excessive amounts (14). Similarly, onions, carrots, cabbage, and unhusked rice were reported

as being devoid of the antineuritic vitamin (26, 27, 220, 133); in contrast, these foodstuffs were said to contain considerable quantities of the water-soluble B (244, 313, 247, 242, 218).

From two test species, represented by the pigeon and the rat, the evidence was highly suggestive that the antineuritic vitamin and the growth-promoting principle were separate and distinct substances, but the limitations of the method, which Mitchell concedes, might lead to an erroneous conclusion; accordingly, too much weight must not be attached to the results. At the time of his writing Mitchell expressed regret that no experiments had been instituted on the antineuritic and growth-promoting properties of the same food sample. Such work has now been done. Levene and Muhlfeld have stated that certain races of yeast which were sufficiently active to maintain normal growth in rats failed to protect polished-rice-fed pigeons from polyneuritis (199).

Similar evidence has been recorded by comparing the anti-neuritic and growth-promoting effects of two yeasts upon the same test species. Preparing like extracts from two strains of yeast, Randoin and Lecoq found that one was able to maintain pigeons and cure polyneuritis, but the other could only furnish maintenance without protecting against the nervous disorder (259). One extract contained two factors, they said, weight-maintaining and antineuritic; on the contrary, the other contained only the weight-maintaining factor. It is interesting that Randoin and Lecoq's results from two races of yeast upon one species of animal paralleled the experience of Levene and Muhlfeld with one strain of yeast upon two species of animals.

Of late, the work upon distinguishing between the antineuritic vitamin and growth-promoting principle has followed the course suggested by the sixth point, *viz.*, the contrasting effect of heat upon them. Perhaps the first attempt in this direction is seen in Emmett and Luros' work (80). Unmilled rice when heated at 120°C. and fifteen pounds' pressure for two to six hours apparently could not prevent the onset of polyneuritis in pigeons, but it did promote growth in rats declining on a vitamin B deficient diet. They concluded that the antineuritic vitamin was destroyed by heating under the designated conditions, but that the water-soluble B vitamin was stable under this treatment. They suggested that the antineuritic factor for pigeons was not the same as the water-soluble B vitamin for rats.

It may be mentioned at this point that their suggestion has been fulfilled, even upon evidence furnished by heat treatment, but with

results unequivocally contrary to theirs. As has now been well established, high temperature destroys one factor of the vitamin, without which the rat fails in growth. In the light of this knowledge, it is puzzling how Emmett and Luros obtained growth in rats fed rice which had been heated for a considerable time at a high temperature. Inasmuch as they fed the rice at such an exceedingly high level, even almost complete destruction of the labile factor could probably not have been detected. The small percentage of the substance escaping inactivation would have been so magnified at the high level that the actual destruction of the greater part would have been masked.

The bios problem. In 1919 research upon vitamin B veered into the field of bios and the cultivation of yeast. From a mycologic standpoint Tanner has so admirably treated the technical disputes waged around the bios question that nothing remains to be said on that score (327). Nevertheless, another side of the subject, the purported relationship between bios and vitamin B, must not be forgotten, because it directed the bent of vitamin B investigation for five years, and because its outgrowth carried an implication in favor of a multiple nature for the vitamin. Under the section of tests in the present article, bios was considered with regard to its lack of identity with vitamin B, a finding which destroyed the value of the yeast test. Here we shall reverse the connection, and indicate how the results from the test cast doubt upon the identity of bios and vitamin B.

Addition of oryzanin, a constituent of the outer layers of natural rice, to fermenting solutions in small amounts greatly accelerated the activities and growth of yeast, according to Kurono's statement published in 1915, but apparently no one gave a thought to associating the yeast growth-promoter with the vitamins (195). Many years before the existence of vitamins for growth was recognized, Wildiers had set forth similar results upon a yeast solution from an unknown organic substance, to which he gave the name bios. Struck with the bearing of Wildiers' work upon the vitamin hypothesis, R. J. Williams found in 1919 that solutions containing vitamin B similarly stimulated the growth of yeast (349). Consequently he postulated that Wildiers' bios might be the water-soluble vitamin B. He then devised a method for comparing yeast growth in the presence of vitamin extracts with that in vitamin-free media. Finding that proliferation in yeast was proportional to the amount of vitamin present, Williams suggested that the method might be used as a test for vitamin B. Not a few investigators

readily seized this proposition, and presented it in various forms as a quantitative test (11, 350, 62, 121, 326).

Naturally the success of the test hinged in part upon its quantitative accuracy, but of greater importance was the question of its specificity for vitamin B. At first glance Williams' data respecting specificity was so truly impressive that the identity of bios and vitamin B could not be other than presumed (349). The substances promoting yeast growth occurred in the same materials as those containing the vitamin. Just as vitamin B was precipitated by phosphotungstic acid and was destroyed at high temperatures, so the yeast stimulatory substance behaved under that treatment. In fact solubility, precipitation, adsorption, heat stability, and resistance to acids and alkalies were alike for bios and vitamin B. These results seemed to justify the inference that the substance necessary for yeast nutrition was identical with vitamin B.

At first Eddy and Stevenson believed that the yeast test needed further standardization before it could possess reliability (60, 61), but later they formulated a method which overcame technical difficulties and which was specific, they said, for vitamin B (62, 63). After applying their method to a study of the properties of the stimulatory substance, they concluded "that the cause of the stimulation of yeast growths in this test was heat-labile, apparently alkali-labile, removable by Lloyd's reagent, and present in Funk's purified antineuritic vitamin mixture." These findings indicated that bios and vitamin B were the same substance. Indeed Eddy and Stevenson stated that their test worked "with small quantities of the Funk antineuritic vitamin as prepared by him," a result which tended "to strengthen the conviction that the antineuritic or B vitamin is the responsible causative agent in stimulating the growth of yeast cells" (164). Miller corroborated the report that heat and alkali impaired the activity of the yeast stimulatory substance, just as they destroyed vitamin B (231).

After adapting a yeast method of their making to quantitative determinations, Funk and Dubin applied the test to a number of such biological substances as purine and pyrimidine bases, amino acids, and extracts of foodstuffs (121). The results indicated that the test possessed great specificity for the vitamin, but Funk and Dubin did not commit themselves. They said: "Some investigators believe that the B vitamine is not identical with the substance promoting growth in yeast. Regarding this contention, we are not prepared as yet to make any definite statement."

In continuation of studies regarding thermal effects upon the water-soluble vitamins, Emmett and Stockholm made comparative tests on vitamin-containing material, after its exposure to high temperatures, in order to determine whether heat had affected its power to increase growth of yeast, to cure polyneuritis in pigeons, or to excite growth in young rats (81). By submitting extracts of rice, heated at 120° for varying periods, to yeast test, they showed that the factor which stimulated growth in yeast was not in the least inactivated by high temperatures. This datum, by the way, contradicted the reports of Williams, Eddy and Stevenson, and Miller (349, 64, 231). As was true in a previous instance (80), rice heated at 120° for two to six hours showed marked alteration again in its antineuritic potency for pigeons (81). Therefore, contrary to Williams' assertion, Emmett and Stockholm concluded that the yeast-growth-promoting factor was not the antineuritic vitamin.

But this inference did not preclude the possibility that the yeast factor and the rat growth principle were identical. Adsorption of a water-soluble B preparation with fullers' earth left a filtrate which was inactive for pigeons; which did not induce growth in rats; but which contained the yeast stimulus factor in quite high concentration. These effects suggested that the yeast principle was not the same as water-soluble B. Thus Emmett and Stockholm concluded that the yeast cell stimulus was not the same as either the antineuritic vitamin for pigeons, or the water-soluble B for rats; but they remarked that whether pigeons or rats required this yeast growth-promoting factor for normal development had not been definitely proved.

Contrary to the findings of Williams, Eddy and Stevenson, and Miller that bios like vitamin B was impaired by heating with alkali (349, 64, 231), Souza and McCollum discovered that vitamin-containing extracts after alkali treatment still exerted a profound stimulating influence upon yeast, whereas they were inactive for rats (309). Similarly, Whipple found that boiling water-soluble B extracts in alkali did not destroy their yeast accelerating activity (347), and Fleming asserted that even *severe* alkali treatment did not decompose the yeast growth principle (87); such treatment was known to inactivate the vitamin. After obtaining stimulation of yeast growth by substances comparatively free from water-soluble B, namely, glucose and amino acids, Souza and McCollum were convinced that all these observations formed "a conclusive demonstration that the use of yeast as a test organism for determining the presence or absence of the antineuritic dietary factor is

complicated by so many disturbing factors as to make it of little if any value."

Then too, MacDonald and McCollum found that yeast grew in nutrient solutions containing no possible source of vitamin B; indeed, that a pure culture grew as well at the end of a series of fifteen consecutive seedings in this solution as it had earlier. They seriously questioned whether yeast was dependent upon a supply of vitamin for continued multiplication; they concluded that either yeast must grow without bios, or it must synthesize the substance to meet its own needs (210). It is well to note that they did not deny the "remarkably stimulating effect of adding various substances such as amino acids and other extractives to such purified nutrients."

When MacDonald and McCollum remarked that extracts of natural foods contained, besides the antineuritic substance, so many other substances "having a favorable influence on the growth of yeast," Ide intervened, as he said, in behalf of his student, Wildiers, and maintained that yeast needs bios and that "small quantities of impurities is an incorrect expression for the bios. It is too soon to answer the question," Ide continued, "whether water-soluble B and bios are the same substance" (170). In reply to this McDonald and McCollum affirmed their previous position, according to which, as Tanner has so well put it, "they were not contending that bios would not stimulate growth but that yeast could grow in a synthetic medium indefinitely, either getting along without it or making it" (327).

Meanwhile, Fulmer, Nelson and Sherwood had shown that the relative potencies of two substances as yeast growth-stimulants could not be determined by comparing the effects of extracts from equal weights of the material. Further, they asserted that water-soluble B was not the growth stimulant in their extracts, since like Souza and McCollum they found that material so treated with alkali that water-soluble B was destroyed still accelerated yeast growth to the same extent as untreated material (97). Moreover, by studying the optimal concentration of each constituent for yeast growth they developed a synthetic medium, free from unknown substances, upon which the yeast grew well for ten months (98). They said that addition of water-soluble B did not improve this particular medium. In this respect they even exceeded MacDonald and McCollum's position that yeast could grow on purified nutrients without the vitamin. Finally, with the synthetic medium at their disposal, Nelson, Fulmer, and Cessna demonstrated that yeast cultivated on a vitamin-free medium could synthesize water-

soluble B, as shown by rat tests (236). As a consequence of these results, they concluded that yeast growth-stimulant was not vitamin B.

That yeast grown on a purified medium can synthesize water-soluble B has received confirmation from MacDonald and McCollum, and Heller (212, 159), although Darrah dissents from this view (43). Eijkman and his associates, and Southgate, on the other hand, said that yeast so growing could not synthesize the antineuritic vitamin; rather the cells absorbed any of the principle available in a medium (75, 308). Randoin and Lecoq implied as much in their view that the biological value of the different races of yeast were different, and depended entirely upon the culture medium (262). These apparently contradictory results gave reason to doubt whether the antineuritic factor and water-soluble B were identical. The matter was not clarified when Funk and Paton stated that yeast abstracted "vitamine D" (Bios) from solution, but left vitamin B (125); Funk and Freedman, however, agreed with Eijkman *et al.* in so far as yeast cultivated on a pure medium could not synthesize vitamin B (126). Opposed to this stand, a number of investigators have reported that yeast, grown on purified nutrients, synthesized the antineuritic vitamin, as shown by the pigeon test (150, 159, 255, 157), so that the bulk of evidence is counter to that of Eijkman and Funk.

Upon comparing the vitamin content of different foodstuffs as determined by the yeast test with their vitamin content as recorded by Osborne and Mendel with the rat method, Williams reported fairly concordant results, but some discrepancies (351). Bakers' yeast by the yeast test showed higher vitamin content than brewers' yeast, whereas animal-feeding experiments gave directly opposite results, namely, brewers' yeast was much richer in vitamin B. He said: "This disagreement between the two methods does not necessarily mean that the substance which promotes the growth of rats fails to promote the growth of yeast. There is evidently some substance which is able to promote the growth of yeast which fails to induce rat growth." This statement was virtually an admission that bios was not identical with water-soluble B. Rather convincingly Hoet showed that certain products, notable among them penicillium medium, could markedly promote yeast growth, but be entirely ineffective for growth of young rats or avian polyneuritis. Therefore he plainly differentiated the substances bios and vitamin B (160).

The climax of interest in the yeast test as a measure of vitamin B had now passed, and one by one the strongest advocates of the method

and of the assumption that bios and vitamin B were the same now abandoned their stand. From studies on the growth curves of yeast in various concentrations after addition of vitamin extracts, and from the finding that the yeast stimulating factor, unlike vitamin B, was not destroyed by alkali, Eddy and his associates conceded: ". . . . that the growth of the yeast cells, as determined by any one of the methods devised to date, is a resultant of so many different factors, mutually interacting, that to interpret these growths as a quantitative measure of vitamin content is unjustified" (65). In the same strain they remarked: "If these factors can be evaluated we believe that it may result in showing that vitamine B plays a part in the action" (66). Finally they said: "It seems to follow that the test is not specific for vitamine B. . . . . On the other hand it seems also to follow that the vitamine B is not ruled out as one of the factors in the stimulation [of yeast]" (65).

It will be recalled that Funk and Dubin had thought that the yeast test, as carried out by their method, was specific for the antineuritic vitamin, yet they had refused to commit themselves (121). Finding now that much larger quantities of fullers' earth were necessary to remove from autolyzed yeast the substance necessary for the growth of yeast, than those which were known to be sufficient for the removal of vitamin B, they said "Our present results show rather conclusively that yeast requires for growth a different substance than that needed by animals, since we were able to separate from autolyzed yeast one substance active for yeast and another for rats and pigeons. . . . . We believe that we are dealing here with a specific substance—either a new vitamine or a cleavage product of antiberi-beri, or vitamine B." This substance, a definite and specific principle stimulating the growth of yeast, which they separated from vitamin B, they called "vitamine D" (Bios).

Resistance to heat and alkali drew a sharp distinction between "vitamines" B and D, according to Funk and Paton; either autoclaving for three hours at 25 pounds' pressure or exposure to alkali impaired the activity of vitamin B, but affected vitamin D much less (125). Substances, either identical with or at least similar to the "vitamine D," which promoted growth of yeast cells, were found in beef, beef-heart infusion, peptone, autolyzed brewers' yeast, and commercial cane sugar (96, 126, 318). That Funk and Freedman assigned true vitamin significance to their D factor was clear from their unequivocal statement that yeast cells are "unable to grow in total absence of vitamine D" (126). They summarized their view in this wise, "The growth of yeast,

therefore, depends on two factors: on the vitamine D found in the medium, and on the amount of vitamine D introduced with the yeast cells used for inoculation." Similarly, from investigation of a score of nitrogen compounds with the yeast test, Willaman and Olsen set forth their opinion that bios was not identical with vitamin B, but that it was of vitamin nature; and that bios was required for normal yeast growth (348). They retained the name bios instead of vitamin D.

From the first MacDonald and McCollum, and Fulmer and Nelson had contended that bios and vitamin B were not identical; with Funk's admission of this fact they were in accord with him just that far. Otherwise they held the sharply conflicting views that bios was not necessary for the growth of yeast, that it did not therefore function as a vitamin, and that it should not be designated "vitamine D." MacDonald maintained that bios was not an indispensable nutrient principle for yeast, since the latter grew in a bios-free nutrient solution and synthesized bios during slow proliferation in such a purified medium (213). She pointed out that bios did not function as a vitamin, because development in an animal is impossible in the absence of the nutritive principles, the vitamins, which higher organisms cannot synthesize for their needs. Likewise, Fulmer and Nelson had been able to cultivate yeast, as has been noted, on a synthetic medium of salts and cane sugar. But Funk and Freedman's assertion that cane sugar contained bios opened the question whether Fulmer and Nelson's successful use of sugar was due to traces of bios. This question received a decisive answer when they employed methose, a synthetic sugar, fermentable by yeast; thus they announced the growth of yeast on a medium wholly synthetic in origin (101).

With this evidence in hand Fulmer and Nelson once more emphasized their position in words that bear repetition. They said: "There are unknown substances which accelerate the growth of yeast. . . . . To this class of substances the generic name "bios" is given. However, bios is not necessary for the growth of yeast, but only makes it grow more rapidly" (102). Characterizing Funk and Dubin's use of the name "vitamine D" for bios as "wholly unnecessary and confusing," Fulmer and Nelson declared that "the term bios has proved satisfactory and, moreover, the name vitamin D should be reserved for any new vitamin that may subsequently be found necessary for the growth and well-being of the animal" (99). Therefore, in the establishment that bios was not a true vitamin the real issue at stake was settled, namely, bios and water-soluble B were not identical.

Just as biochemists have been interested in the isolation of vitamin B, so they have engaged in elucidating the chemical nature of bios (90, 91, 92, 322, 319, 265, 266). Complications, however, entered the problem when Fulmer, Duecker and Nelson revealed evidence that bios was not a single substance, but that it comprised at least two different yeast growth stimulants (100, 103). This multiplicity has been generally confirmed (232, 352, 185). Lash Miller supported it in his account of the fractionation of the yeast growth-stimulant into two substances, Bios I and Bios II, neither of which alone was active, but both combined possessed the bios effect (232). His associates have since carried the work to further completion (209, 55, 56, 306). With the two partially purified principles, Bios I and Bios II, available, Deas was afforded an opportunity to establish almost conclusively whether or not bios was identical with vitamin B. Her answer was decisively in the negative, since Bios I, Bios II, or both would not induce growth in rats (45).

Almost simultaneously with Miller's report, Eddy, Kerr and Williams announced the isolation of *a* bios in crystalline form. Concerning the multiplicity view, they said: "Nothing in our method of isolation negates the possibility of more than one bios. In fact, the relative stimulation producible by the addition of our pure product and by use of autolyzate [of yeast] lends strong probability to the suggestion that the latter contains more than one growth stimulant" (67, 184). This probability was apparently borne out in Kerr's very recent report that yeast autolyzate yields three bios-containing fractions: α-bios, the substance just described; β-bios, hitherto unknown; and γ-bios, suggestive of Miller's bios II (185). Zajdel and Funk have stated that they could not repeat Eddy, Kerr, and Williams' work on the isolation of a bios (363); and Lucas experienced difficulties with Eddy's yeast cultures and bios (209), which has, however, received an explanation (214).

In retrospect, it is manifest to what devious paths leads a faulty premise, in this instance an assumption that bios was vitamin B. If the recital has seemed a digression, it is no more so than the actual events. After voluminous work had established the falsity of the assumption, vitamin B research returned almost to its original position, but not quite. Even after bios was shown to be different in nature from vitamin B, the suggestion would not down that it was related in some way, however remote, with the vitamin. As a consequence, bios investigation stimulated thought upon the multiplicity of vitamin B.

Recent developments. By fractional adsorption with fullers' earth or norite, Funk and Dubin were able to effect, as has been noted, an almost

quantitative separation of vitamin B, which was curative for avian polyneuritis, and of another substance, designated by them "vitamin D," which was specific for the stimulation of yeast growth (123). At that time they stated, "Although vitamin D has been obtained free from vitamin B, as far as our animal experiments have shown, the reverse is not true. It is evident, therefore, that most animal tests conducted up to the present were carried out with a mixture of vitamins B and D."

The implication which this statement conveyed became clear when they tested the two preparations on rats and on pigeons (122). They reported: "While the pigeons were found to need only the vitamine B when fed on a vitamine-free diet, the rats exhibited a somewhat different behavior. . . . . The rats receiving the vitamine B or D fraction as an addition grew only for a few weeks at a slow rate and started to die out after two months. While increasing the amount of one vitamine did not have any effect, the addition of the missing component in both of the above cases caused a prompt resumption of growth. The rats given both B and D from the start together with the last filtrate, which contains neither B nor D, showed a normal behavior both in regard to growth and appearance. . . . . The results suggest that the rats and possibly other mammals require, besides the vitamine A, at least two vitamines of the B type, namely the B and D vitamine, for their well-being and growth."

Questioning the use of the rat as a test animal for purified fractions of vitamin B, Funk and Paton said, "The rat serves well the purpose when natural foods are tested for vitamine B, plus the other nutritive factors which regularly accompany this vitamine, but fails partially where purified vitamine B, devoid of vitamine D and perhaps other vitamine, is administered" (125). The different requirements of the pigeon and of the rat for vitamin B are again emphasized in the work of Funk, Harrow and Paton (127). After testing extracts obtained from yeast and rice-polishings by various solvents, they said:

> The hope that a single solvent would show a selective affinity for either vitamin B or vitamin D, or that a particular solvent would yield an extract that would induce growth in pigeons and in rats (or perhaps *vice versa*) has not been realized. . . . . Every extract that showed itself active for pigeons was also active for rats. This, however, is not true of the residues, for here a number exert positive influence upon rats, and a negative one upon pigeons. The reverse is not true; in no case is there a positive response from pigeons that is not accompanied by a positive one from rats. This permits of a number of speculations. May we, perhaps, assume that the vitamin for rats is less easily soluble in the solvents used than the vitamin for pigeons?

In like manner Laird tested a series of vitamin B preparations in parallel upon the pigeon and upon the rat (196), and found that some preparations giving positive tests with the rat were negative with the pigeon, and vice versa (196). Since Plimmer and Rosedale had declared that pigeons in comparison with several birds had the least vitamin B requirement (256, 255), Laird suggested that in instances where the test with pigeons was positive but the test with rats was negative, it was possible that the amount of vitamin B in the preparation was too small for the latter animals. No explanation, however, for the positive results with the rat and negative with the pigeon could be furnished on this basis. Laird did not apply Funk's interpretation of two vitamins to his results. Instead he remarked, "It is impossible to decide, on the basis of existing data, as to whether or not what is now known as vitamin B is a single substance or is of multiple nature."

Upon the basis of difference in distribution Heaton agreed with Funk that deprivation of the water-soluble B vitamin from rats involved a double deficiency of two distinct substances: in one case the antineuritic vitamin, in the other the yeast activating substance, variously designated as bios or "vitamine D" (158). "There are two water-soluble vitamins," said Heaton, "present in yeast: it is the absence of one of these, vitamin B, which causes polyneuritis in birds; while the other. . . . . is the bios of Wildiers [vitamine D of Funk and Dubin or biocatalysator of von Euler (82)], necessary to the activity of low concentrations of yeast cells; both of them are necessary for the proper growth of rats. The prevailing uncertainty as to the identity of the antineuritic and the growth-promoting vitamins is due, therefore, to the fact that the latter includes the antineuritic substance, but contains also another essential factor, the vitamin D."

Tönnis, on the other hand, maintained that a critical study of the rat's vitamin requirement and of the data from which the existence of an additional vitamin had been inferred, did not justify the assumption of a "vitamine D" as differentiated from vitamin B (329). By the latter term he meant only one substance, possessing antineuritic and growth-promoting properties. Gaglio swung to the other extreme in his statement that postulation of only one specific antineuritic principle was indefensible inasmuch as many different substances exerted antineuritic properties (131). Of the water-soluble vitamins Kinugasa and Hattori mentioned three forms: one for polyneuritis, another for yeast growth, and a third for rat growth (189). It is interesting that Scotti-Fogliene listed the pigeon as needing two vitamin B factors (281, 282,

283, 284). Upon autoclaving a rice solution he condensed the escaping vapor at a desired temperature and obtained two fractions: the distillate and the residue. The one fraction, which had been driven from the rice between 120–134°, gave no Folin reaction; the other, which remained bound at high temperatures, responded with the color test. Each by itself was inactive, but together they cured polyneuritis in pigeons. Vitamin B, he said, consisted of two substances.

Even more significant was Špinka's demonstration that an antineuritic factor could be separated from a growth-promoting principle, both of which were needed by the pigeon (310, 311). He prepared a product containing only the first factor, vitamin B, since it cured polyneuritis but did not prevent alimentary distrophy in birds; addition of the missing component, named vitamin D, brought increase in body weight. Perhaps this finding explains Seidell's comment that his silver vitamin compound prevented development of polyneuritic symptoms in pigeons, but did not protect them against loss in weight "possibly as a result of the absence of growth-promoting principle in the purified antineuritic product" (288). Such a denouement was again forecasted in his words: "It is, of course, a question whether the antineuritic activity resides in a single member of a mixture or is a function of the complementary action of two or more individuals. The universal experience is that no single isolated compound has ever been found to exhibit unmistakable antineuritic activity" (291).

All the foregoing evidence which pointed to the multiple nature of vitamin B was a worthy forerunner for the conclusive demonstration which was to follow. Because the vitamin needs for growth were presumably greater than those for protection against polyneuritis, and because it was by no means certain that the rat did not require more components than the pigeon for growth and well-being, biochemists were exceedingly desirous of ascertaining the exact number of substances in vitamin B necessary for the rat. Apparently incidental to work in another direction, as so often happens, Smith and Hendrick presented the decisive evidence which revealed the relation of water-soluble B to rat growth (307). In attempting to remedy the deficiency of a diet composed of rolled oats, casein, vitamin A and inorganic salts, they found that a small addition of dried brewers' yeast gave a much better growth curve. It was not clear whether the improvement was due to yeast protein, water-soluble vitamin, or to some unknown factor. In attempting to rule out vitamin B they supplemented the basal ration with dried brewers' yeast, autoclaved for 6 hours at fifteen pounds, which

procedure, as was shown by tests, completely destroyed vitamin B. Rats responded with growth. Therefore, it was manifest that autoclaving brewers' yeast, though destroying its vitamin B content, did not impair its efficiency in supplementing the oat deficiency. That the oat kernel was not deficient in vitamin B but in some other factor, which was present in brewers' yeast and which withstood prolonged autoclaving, was the logical conclusion.

Furthermore, Smith and Hendrick properly inferred that casein was deficient not only in vitamin B but also in the second factor, else it would have supplemented the oats. Experiments bore out their reasoning. A basal diet containing salts, cod-liver oil, and casein as source of protein, when supplemented with Seidell's picrate as the source of vitamin B, did not check decline in rats. An addendum of autoclaved yeast (in which vitamin B was destroyed) to the basal diet likewise did not prevent failure. But the addition of both Seidell's picrate in small dosage and autoclaved yeast resulted in good growth. Again evidence was on hand that yeast contained, besides vitamin B, a hitherto unrecognized factor which was resistant to high temperature in the autoclave. Both were growth essentials. If Seidell's picrate was added to the basal ration in sufficient dosage, fair growth ensued. Some of the unrecognized factor obviously was present with the antineuritic vitamin in this concentrate. By demonstrating that a synthetic ration with casein as the sole source of protein must be supplemented with two vitamins of the B type, *i.e.*, the unknown thermostable factor and vitamin B, in order to become adequate for the rat, Smith and Hendrick convincingly established the multiple nature of growth-promoting water-soluble B.

Previous to the foregoing work Goldberger and Tanner had expressed the view that fresh meat and milk contained a factor which was essential to the prevention of pellagra (134). This they called factor P-P (135). After the appearance of Smith and Hendrick's paper, Goldberger and his associates communicated results which confirmed the existence of two factors, and which by reason of additional facts shed much light on the subject (136). They stated that pellagrous patients fed a "yeast vitamine" powder, *i.e.*, a concentrate, recovered from pellagra but in some cases developed beriberi. This yeast concentrate therefore contained not only vitamin B, but also the pellagra-preventive P-P, the latter being present apparently in greater amount.

Assuming that black tongue in dogs was the analogue of pellagra in man, since both responded favorably to the same treatment, they postulated that the dietary essential P-P was just as primarily concerned in

the prevention of the one disease as in the prevention of the other. Accordingly, Seidell's activated solid contained, in addition to vitamin B, the factor P-P, since it prevented black-tongue in dogs. Then, too, factor P-P was evidently adsorbed, as was the antineuritic vitamin, from an aqueous extract of yeast by fullers' earth. At once it became imperative to ascertain the relation of factor P-P to the vitamin B. Inasmuch as the belief was prevalent that the water-soluble vitamin B contained at least two distinct dietary essentials, one the antineuritic vitamin (vitamin B *sensu stricto*) and the other a growth-promoting factor, which Funk believed was identical with bios, it was necessary to correlate factor P-P to these two principles at least.

Previous work had shown that beef, which was poor in the antineuritic vitamin, was effective against pellagra; therefore vitamin B *sensu stricto* was excluded from consideration in the prevention of this disorder. Similarly, patients taking substances which cured pellagra often developed beri-beri, for which the antineuritic vitamin was specific. From these two lines of evidence, it followed that the antineuritic principle and factor P-P were separate and distinct substances. Now, with respect to the relation of factor P-P to the second, or so-called growth-promoting essential, Goldberger and his co-workers were obliged to use the rat test. They had already found that yeast autoclaved at fifteen pounds for two and one-half hours retained much, if not all, of its activity for the prevention of black tongue in dogs. But, like Smith and Hendrick, they found that autoclaved yeast fed to rats as the sole source of water-soluble B, arrested their growth and led to loss in weight and polyneuritis. On the contrary, unheated yeast at a much lower level was sufficient for good growth. Thus autoclaving inactivated the water-soluble vitamin, but had no effect upon the P-P factor.

Obviously, however, since rats failed on autoclaved yeast, the factor P-P was not in itself growth-promoting. From this outcome it seemed that if the so-called growth-promoting water-soluble vitamin of yeast was distinct from both the antineuritic and the P-P factors, then either the heating had inactivated it, or like factor P-P, it was not a special "growth" factor. But that actually factor P-P or some associated and relatively thermostable factor, distinct from the antineuritic principle was essential in addition to the antineuritic factor for growth in the rat soon appeared.

When autoclaved yeast was added at 40 per cent level to a basal diet complete except for lack of water-soluble B, rats declined in weight

and died with or without polyneuritis. Similarly, when an 85 per cent alcoholic percolate of maize meal was the supplement to the basal diet, the rats after a slight initial gain soon lost weight. But if the basal diet was supplemented both by autoclaved yeast at 10 per cent level and maize meal percolate at 5 per cent level, good growth ensued.

Again, when the same basal diet was supplemented with 20 per cent dried lean beef, which contained factor P-P, rats ultimately declined and died. In some experiments when signs of polyneuritis appeared in rats on the basal ration plus lean beef, the inclusion in the diet of as little as 5 per cent maize meal percolate, which as the sole source of water soluble vitamin at 40 per cent level would not permit growth, now produced sharp response in the animals. Evidently maize meal percolate contained an essential curing polyneuritis in the rat. And although not growth-promoting of itself, this antineuritic principle permitted growth on a diet adequate except for water-soluble B when combined with suitable proportion of a factor in autoclaved yeast or beef, probably P-P, which itself could not promote growth.

In summarizing the relation of factor P-P to water-soluble B, Goldberger and his co-workers had this to say: "First, that the antineuritic factor (vitamin B *sensu stricto*) is distinct from the factor P-P and does not in itself suffice for the growth of the rat; second, that if the term 'water-soluble B' includes, as some investigators have suggested, in addition to the antineuritic factor a so-called growth-promoting essential (possibly identical with Wildiers' bios), this, like the antineuritic factor, is either inactivated by autoclaving, or does not suffice by itself, for the growth of the rat; third, that factor P-P or some associated, and, in yeast, like P-P thermostable factor (possibly the so-called growth-promoting factor) distinct from the antineuritic vitamin, though not sufficing in itself for the growth of the rat, is, in combination with the antineuritic, essential for growth in rats." Thus Goldberger and his associates confirmed Hendrick and Smith's statement that there are two factors in water-soluble B, both of which are necessary for the rat. And Goldberger *et al.* went a step further by showing the relation of their factor P-P, which was apparently the same as Smith and Hendrick's thermostable factor, to the two factors in water-soluble B.

From the preceding work it was established that maize extract and Seidell's activated solid contained little of the factor P-P, which was their limiting factor since they both contained sufficient antineuritic vitamin. Rats, when given the maize extract or activated solid at low level, not only suffered loss in weight but also frequently, for want of factor P-P,

developed a pellagrous condition, characterized by depilation, dermatitis, buccal lesions, and gastro-intestinal disturbance. Inclusion of the missing P-P factor in the diet was followed by disappearance of these symptoms and by resumption of growth.

Although the facts on the whole made it probable that this thermostable, fullers' earth adsorbable factor, which was seemingly specific for the pellagra-like condition in the rat, was the same as the factor needed for pellagra in man and for black-tongue in dogs, and in combination with the antineuritic factor for growth in the rat, Goldberger and Lillie reservedly said: "The possibility, remote though it seems, is not excluded that there may be in yeast more than one such thermostable factor which further study may succeed in differentiating. Moreover, it is not clear that all essential factors, or necessary relations among such factors, for the nutrition of the albino rat have as yet been determined" (137). Recently Goldberger and Wheeler reported that the pellagra-preventive factor P-P is present in commercial wheat germ. Since this food material was effective against black tongue in dogs as well as against pellagra in humans, their hypothesis that the two diseases were analogous was strengthened (138). Further, inasmuch as wheat germ was known to contain the antineuritic factor, their demonstration that it also contained P-P lent essence to the view that "water-soluble B" could be resolved into the two factors.

After advancing their hypothesis that pellagra in man, pellagra in rats, and black-tongue in dogs were analogous, Goldberger and his associates depended, it would seem, upon evidence from cure of black-tongue in order to properly relate their P-P factor to the water-soluble vitamin. More specifically, autoclaved yeast prevented the canine disease, but it did not afford growth in rats. Autoclaving had undoubtedly destroyed water-soluble B, but not P-P; hence, the two factors were not identical. Admittedly this is not *sine qua non* in the thread of their argument; if it were,—if their explanation of the relation of P-P to vitamin B stood or fell by this point—we should be at loss to appraise the situation, because at almost every turn their pronouncements with respect to black-tongue in dogs have been countered by opposite reports from Underhill and Mendel.

By means of a diet comprising boiled peas, cracker-meal, and cotton-seed oil, Chittenden and Underhill in 1917 described the appearance in dogs of a pathological condition which was regarded as closely resembling human pellagra (30a). Four years later, Wheeler, Goldberger, and Blackstock pointed out the striking similarity of this pellagra-like

syndrome to black-tongue in dogs (346a). If these two conditions were identical, black-tongue in dogs became presumptively the analogue of pellagra in man. Not content with basing this contention solely upon the similarity in pathology of the two diseases, Goldberger and co-workers gathered evidence which showed that both black-tongue and pellagra responded favorably to the same foodstuffs. Yeast and lean beef muscle possessed therapeutic and preventive value not only for black tongue, but also for pellagra in humans (135, 135a, 136).

Meanwhile Underhill and Mendel had discovered that the Chittenden-Underhill syndrome in dogs was associated with a lack of some unknown constituent in butter-fat, since this foodstuff would prevent or cure the disorder (333a). Inasmuch as cod liver oil exerted no beneficial effect, the unknown principle was not vitamin A. The protective substance in butter-fat deteriorated with age, and singularly a diminution in color accompanied this loss in activity. Impressed by the relationship between the color and potency of butter, these investigators studied the pigments imparting to this foodstuff its characteristic color; namely, the carotinoids. In carrots, as in butter-fat, carotin is predominant, and it is present to some degree in egg-yolk. Showing physiological activity parallel to its carotin content, egg-yolk conferred upon dogs some protection, but it was not so effective as butter-fat. Carrots, however, were particularly active in alleviating the Chittenden-Underhill syndrome. Underhill and Mendel were so impressed by the correlation that they adopted "as a working hypothesis the possibility that the effective agent is either a natural pigmentary substance or some unidentified compound associated therewith."

Using diets associated with the occurrence of pellagra in humans, Goldberger and Wheeler induced in dogs a condition which clinically and pathologically was indistinguishable from black-tongue, and which also was regarded as identical with the Chittenden-Underhill pellagra-like syndrome. Aside from the fact that black-tongue had resulted from pellagra-producing diets, the appearance of dermatitis in the dogs furnished almost conclusive evidence, they stated, that black-tongue in dogs and pellagra in humans were identical (138b). But Goldberger's assumption of the identity of the Chittenden-Underhill syndrome in dogs, black-tongue in dogs, and pellagra in man presents several irreconcilable aspects. Whereas Underhilll and Mendel had found butter-fat extremely effective for the Chittenden-Underhill syndrome (333a), Goldberger and Tanner reported that this foodstuff failed to prevent pellagra in humans and black-tongue in dogs (134, 136). Again, Under-

hill and Mendel had obtained excellent results with carrots in the cure of the canine syndrome; but Goldberger and colleagues stated that carrots did not prevent pellagra in patients, and exerted only slight effect in black-tongue experiments, an outcome indicating that the necessary factor was present only in relatively small amount (138a). According to Goldberger and associates, their results were not necessarily inconsistent with those of Underhill and Mendel, for considerable quantities of carrots had to be ingested to produce beneficial effects.

The findings of the two groups, nevertheless, have recently drawn farther apart. For example, Goldberger had found yeast effective against black-tongue and human pellagra; Underhill and Mendel, on the contrary, have now recorded that yeast does not protect dogs against the syndrome (333b). At the same time the latter investigators reaffirmed their stand that a relation exists between carotinoids and their factor, since butter-fat, egg-yolk, and carrots are curative for the pellagra-like condition. This assumption was seemingly borne out by their assertion that crystallized carotin in relatively small doses is capable of curing the canine disease. They believe that naturally occurring pigmentary substances in foodstuffs play a rôle in nutritive processes.

Inasmuch as substances possessing black-tongue preventive action appeared effective against pellagra, and those that failed against black-tongue were likewise ineffective against pellagra, it cannot be denied that Goldberger had reason to believe that the two diseases were identical (136). His factor P-P, as a consequence, would be concerned in the prevention and causation of both diseases. But his inclusion of the Chittenden-Underhill syndrome as an analogue of both black-tongue and pellagra presents a formidable problem because of his finding that yeast cured black-tongue and pellagra but butter and carrots did not; whereas, Underhill and Mendel have maintained that butter and carrots cured their syndrome but yeast was without effect. If the two groups of investigators are not dealing with the same nutritional factors and with the same canine diseases, despite clinical similarity, it is interesting to speculate which condition in the dog is really analogous to human pellagra, since each has been described as pellagra-like. The only available evidence has come from Goldberger; namely, that black-tongue is cured by the same substances as pellagra, while butter, which is effective against the Chittenden-Underhill syndrome, does not prevent pellagra in patients. Of the antithetical positions Underhill and Mendel say: "The discrepancies between the results of Goldberger and his associates and our own are so great as to prohibit attempts at explana-

tion" (333b). But they pointed out that the diets used in each case were different, a fact to which some significance may be attached.

To return to the discussion of the multiple growth factors, Goldberger and associates reminded investigators using the rat-growth test in the isolation of vitamin B that the two essentials (vitamin B *sensu stricto* and P-P) at least must be taken into account. To determine the actual activity of a pure concentrate, it would have to be combined with one or the other missing factor, otherwise it might appear inert. Subscribing to this warning, Seidell pointed out that so far as growth in pigeons was concerned, autoclaved yeast contributed no appreciable effect to a basal diet of polished rice and his vitamin picrate (297); whereas, Smith and Hendrick had already recorded that autoclaved yeast as well as Seidell's picrate was necessary to supplement a basal ration for growth in the rat. It was patent that the rat used, besides the vitamin concentrate, some element of autoclaved yeast which was not required by the pigeon; accordingly, the nutritive needs of the pigeons, as anticipated, were simpler than those of the rat.

Interpreting the growth essential, vitamin B, as constituting an antineuritic vitamin and another active element, Seidell explained that the pigeon needed only a part of what is commonly considered vitamin B. Therefore the choice of the test animal in isolation work would depend upon the particular constituent of the complex under investigation; the rat would be suitable for vitamin B study, while the pigeon would be more appropriate, he stated, for an index of antineuritic vitamin. Correspondently, Chick and Roscoe stated that Peters' alcoholic extract of activated charcoal would not induce growth in rats, although it was described as highly potent for the cure of polyneuritis in pigeons (30). When autoclaved yeast was included with the concentrate, however, the rats responded in growth.

Confirmation of the work of Goldberger *et al*, likewise, came from Chick and Roscoe (30). Rats fed a basal ration supplemented with an 85 per cent alcoholic extract of purified wheat embryo, as a source of antineuritic factor, did not grow, but contrary to the behavior of control rats on the unsupplemented basal diet, they showed no signs of collapse at the end of three or four weeks. After six to eight weeks the animals on the test ration sometimes died without showing external symptoms; in other instances, they developed at this period the condition described by Goldberger as pellagra. Although in poor condition, the rats maintained weight and survived for two to three weeks with the pellagrous symptoms.

Again, if rats near collapse at the end of three to four weeks on the basal diet were given Peters' extract or an 85 per cent alcoholic extract of wheat embryo (sources of the antineuritic vitamin), they showed sharp response in growth for three to four weeks, at which time they began to decline and to develop the pellagrous condition. Resumption of growth and cure of pellagra was effected at this point by addition of autoclaved yeast. Of particular importance is this experiment, since it has been the common practice of investigators in testing the activity of water-soluble B concentrates to watch the effect for a period of four weeks at most. If the rats grew, the test was considered positive and was discontinued. But Chick and Roscoe have shown that a debilitated rat may respond sharply to a preparation for three to four weeks, only to ultimately fail from the want of one factor. Again is emphasized the misleading nature of short experiments. It would seem that before pronouncing a preparation as positive, investigators should follow the response of the rat for at least eight weeks. Negative experiments due to lack of both factors would become manifest much sooner since the animals would probably not survive for this duration.

According to Chick and Roscoe, neither autoclaved yeast nor Peters' yeast extract given separately in a basal ration could promote growth, but respectively they could relieve the pellagrous and neuritic symptoms. Together they promoted growth. Obviously native yeast contained both principles which were necessary for growth: the one, the antineuritic vitamin was destroyed in the autoclaved yeast, the other, the P-P factor (called vitamin B *sensu stricto* by Chick and Roscoe) was not present in Peters' yeast concentrate. Concerning the presence of both factors in wheat embryo, they stated that "specially pure" embryo, supplemented with autoclaved yeast at a low level, yielded good growth; on the contrary, Peters' yeast concentrate (source of antineuritic vitamin), supplemented with autoclaved wheat germ, gave subnormal growth. These results indicated that wheat embryo, although a rich antineuritic source, was poor in P-P factor.

Where Smith and Hendrick, and Goldberger *et al.* firmly laid the groundwork for the multiple nature of vitamin B, these several investigators have already contributed in one way or another to the superstructure. Notwithstanding that workers have approached the problem from diverse angles, their findings have invariably converged to the same conclusion, that vitamin B comprises at least two substances. For instance, according to Hauge and Carrick, dried brewers' yeast as the sole source of vitamin B in a diet permitted rapid growth in

chickens until the tenth week, but it could not prevent the appearance of polyneuritis (156). In other experiments, ground corn as the vitamin B source, afforded protection against polyneuritis, but it induced only very slow growth in the chickens. On a diet containing a mixture of the yeast and of the corn, the chickens grew rapidly, and escaped polyneuritis. These results suggested that the corn was rather rich in the antineuritic substance, but relatively poor in the growth-promoting substance; on the contrary, the yeast appeared to be very rich in the growth-promoting substance, but relatively poor in the antineuritic substance. It was apparent that the antineuritic substance, though supplied in abundance, would not promote rapid growth, and conversely that a diet might be capable of promoting rapid growth without preventing polyneuritis. Naturally, Hauge and Carrick concluded that the antineuritic vitamin and the water-soluble growth-promoting vitamin were not identical.

Comparative tests by Salmon, using pigeons, on the seeds of the velvet bean and of the soy bean, and with leaves of the velvet bean and of the rape plant showed a higher antineuritic value for the seeds than for the leaves (268). But tests on rats showed that the leaves were more potent than the seeds in their growth-promoting action. By means of fullers' earth he obtained from velvet bean leaves two fractions, which separately were inactive but together induced growth. From these results he reiterated the view that vitamin B was a complex consisting of at least two substances: a specific factor, vitamin B-P, which prevented polyneuritis in pigeons and rats, but was not of itself growth-promoting; a second factor, probably P-P, which did not prevent polyneuritis or of itself promote growth, but which was a very potent growth-stimulant when added to the antineuritic factor.

Lately, Salmon and his associates asserted that fullers' earth adsorbed both the B-P factor and the P-P factor of the vitamin B complex, but that under the proper conditions the former factor was more completely adsorbed than the latter (269). Availing themselves of this finding, they removed vitamin B-P as completely as possible from an extract containing both factors, and inactivated any remaining traces by heat. The resulting solution, rich in factor P-P, was concentrated according to the Osborne and Wakeman method, thereby yielding a fraction which prevented pellagra-like symptoms and which accelerated growth when fortified with sufficient B-P factor. In growth experiments, they stated, a low concentration of either vitamin B-P or P-P may be the limiting factor.

Agreeing that two vitamins were essential to growth, Sherman and Axtmayer pointed out that neither individually met the primary conception of vitamin B, so that it was not appropriate to assign this letter to either factor into which the complex had been resolved. They selected the designation vitamin F (rather than vitamin B or B-P) for the antineuritic factor, and the term vitamin G (rather than vitamin B or P-P) for the pellagra-preventive principle (303). Using ground whole wheat, autoclaved yeast, and dried skimmed milk as experimental food materials, they found that each separately gave poor growth. By various combinations of these foodstuffs they were able to show the relative content of each in the two factors. For instance, autoclaved yeast satisfactorily supplemented the wheat, a result which showed that wheat was relatively rich in vitamin F (antineuritic), and was limited in its apparent vitamin B value by its low content of vitamin G. Native yeast, of course, proved rich in both factors, but autoclaving destroyed most, probably not all, of vitamin F, leaving the product relatively rich in vitamin G. Milk and wheat supplemented each other for growth, whereas milk and autoclaved yeast did not. Milk, therefore, was relatively richer, they said, in vitamin G than in vitamin F, and the latter was the limiting factor in the vitamin B complex of this foodstuff. The results from these studies on the supplementary relations of growth-promoting foodstuffs were in harmony with the conception of a multiple nature for vitamin B.

A highly purified diet of casein, salts, sucrose, lard, and cod liver oil became satisfactory for growth upon addition of yeast, according to Evans and Burr (85a). Apparently yeast contained all the vitamin B factors necessary for growth. When, however, the highly purified basal diet was supplemented with the antineuritic concentrate, tikitiki, a dilute alcoholic extract of white rice polishings distributed in the Philippines, only the slightest growth took place. On the other hand, a more impure basal diet of commercial casein, salts, corn-starch, lard, and cod liver oil, upon inclusion of tikitiki, showed very considerable growth. Tikitiki, manifestly, was almost entirely lacking in growth-promoting vitamin B, though possessing the antineuritic vitamin. Subsequent investigation showed that the successful supplementing of the impure diet by tikitiki was due to the presence of the growth-promoting factor in the commercial cornstarch and in the commercial casein. Further evidence for multiplicity issued from Rosedale's preparation of a concentrated extract of rice polishings which not only cured polyneuritis but also produced a steady growth in pigeons. Addition of lead acetate to

this solution produced a precipitate which possessed no antineuritic activity, but could stimulate growth in the birds (264).

Nor has the number of substances constituting vitamin B been limited to two. From parallel experiments upon pigeons and rats in which the same supplements were administered to each, Williams and Waterman expressed the view that young rats required two vitamin B factors, but one of these, they said, was of little or no consequence to the pigeon (362). On the other hand, pigeons required a substance not needed by the rat, since normal weight of pigeons was not maintained on a diet that supported normal growth in rats. This third unknown factor required by pigeons but not by rats was present in whole wheat. Beyond this Lecoq has provisionally postulated the presence in yeast of four different vitamins of the B type: the antineuritic vitamin of Funk, the nutritive vitamin of Randoin and Lecoq (258, 260, 305, 261), the antipellagrous vitamin of Goldberger *et al.*, and the yeast growth-promoting vitamin of Funk and Dubin. He stated that the pigeon normally required the addition of the four vitamins; whereas the rat required only three, the first two being used interchangeably (195).

From a series of experiments upon mammary secretion in rats, Hartwell reached the conclusion that a high protein diet created a need in the lactating female for high intake of vitamin B, else the ration was not adequate for normal milk production (151, 152, 153, 154). The relationship between protein and vitamin B was quantitative in that corresponding increases between the two resulted in no ill effects. In harmony with this idea Drummond and his colleagues stated that diets consisting largely of protein proved inadequate for growth in young rats until the proportion of vitamin B was raised (51, 263). Increasing the level of yeast in the ration, for example, offset the untoward effects of the excessive amount of protein. Supplementing the high protein diet with yeast autoclaved in strongly alkaline medium, a treatment known to destroy vitamin B, did not furnish growth. Similarly, increasing doses of Seidell's concentrate did not enable the rats to overcome the handicap of the diet rich in protein. But the addition of both the alkali-treated yeast and Seidell's concentrate to the high protein ration permitted good growth in the rats. These results indicated that there were two substances in yeast which were responsible for its vitamin B action and which played a rôle in regulating growth in young rats. These factors could be differentiated by the action of hot alkali which destroyed one, apparently the antineuritic vitamin, and which left the other, which was primarily concerned in rendering high-protein diets

adequate for growth, still active (155). It is interesting to speculate upon the relation of Hassan and Drummond's alkali-stable factor to Smith and Hendrick's thermostable factor. Sherman and Gloy, it may be said, find no basis for the belief that the vitamin B requirement of the rat is influenced by protein intake (302).

In work similar to that of Evans and Burr in nature, Kennedy and Palmer showed that a highly purified ration, presumably complete except for vitamin B, (249, 250) became adequate for growth of rats by addition of yeast, but that it remained deficient when the source of vitamin B was either the Osborne and Wakeman concentrate from pure dry yeast, or the residue left from the preparation of this concentrate, or a commercial yeast concentrate, or dry yeast which had been autoclaved two and one-half hours at fifteen pounds pressure (182). Further, either alcohol-extracted yeast, or the residue left from the same kind of yeast after the removal of the Osborne and Wakeman vitamin fraction, or yeast that had been autoclaved to destroy its growth-promoting and antineuritic properties, so supplemented a non-growth-promoting ration, already containing an adequate amount of vitamin B in the form of wheat embryo extract, that average growth was attained. In contrast to this distribution, neither the Osborne and Wakeman concentrate nor an alcoholic extract of yeast carried adequate amounts of the growth factor which was present in the three sources: the alcohol-extracted yeast, the residue left after removing the Osborne and Wakeman concentrate, or autoclaved yeast. The growth factor with which they were dealing was stable to high temperatures in the autoclave; it was only slightly extracted from yeast by alcohol or acidulated water. In some respects, they stated, this factor was similar to the P-P factor, but the two were not identical. They concluded that yeast contained some factor other than vitamin B (antineuritic vitamin) and that this factor was necessary for satisfactory growth.

Again, their highly purified ration containing an alcoholic extract of wheat germ as the source of vitamin B was supplemented for good growth when consumption of excreta was permitted (183). But the excreta, extracted with 90 per cent alcohol, could no longer supplement this ration. Apparently the excreta contained an unrecognized factor which was removable by 90 per cent alcohol. That it was not vitamin B was certain because the ration contained a sufficiency of this essential in the wheat germ extract. Whether the factor in the excreta was related to the factor found in yeast after the removal of the alcohol-soluble vitamin, they were not prepared to state, although their data at that time did not point to an identity.

From time to time evidence has been presented of the existence of additional water-soluble factors, the relation of which to vitamin B was highly suggestive but never conclusive. Particularly in this category may be mentioned Emmett and Luros' growth factor carried by lactose (78, 79). Then too, Boas stated that certain foodstuffs contained a protective factor X, which possessed the power of counteracting the ill effects resulting from the ingestion of dried egg white (18, 19, 20). This factor showed a similar distribution in many ways to that of the water-soluble B vitamins; it was not identical, however, with either the antineuritic factor or the P-P factor.

During the past year Fridericia and co-workers called attention to a curious phenomenon which occurred spontaneously among young rats fed a basal diet devoid of vitamin B. Out of a number of animals a few responded with growth and remained normal for more than two generations, notwithstanding the absence of the vitamin from their food (96a). Instead of the anticipated onset of decline and of polyneuritis, all symptoms of vitamin B deficiency disappeared, and simultaneously the feces became white and bulky. The phenomenon was called refection. Once started it could be transmitted artificially by feeding the white feces from refected rats to young animals which had been restricted to a diet lacking vitamin B. The transmitted condition lasted for months or years.

The whiteness of the feces from refected rats was not due to an absence of bile pigments, but to a large content of unchanged starch grains. The white feces contained more amylase than the feces from non-refected rats. This amylase, moreover, could dissolve native grains of rice starch, but it could not act to any appreciable extent upon the starch contained in the white feces. The resistance of the latter starch to the action of amylase appeared to be due, we are told, to some protective substance, adsorbed upon the starch grains in the feces. This substance was soluble in acid alcohol. White feces of refected rats, furthermore, contained considerable amounts of vitamin B; while feces from non-refected rats on a water-soluble vitamin-free diet contained very little of vitamin B. Finally, the bacterial flora and the chemical reaction of cecal contents of refected rats were different from those of non-refected animals.

Thus refection seemed to manifest two different effects: the first, growth and prolonged maintenance of health without vitamin B in the food seemed to depend upon the production of vitamin B in the intestinal tract of refected rats; the second, the production of white, bulky feces,

was due to a defect in the digestion of starch. The connection which might exist between these two effects was not apparent. Some relation, nevertheless, seemed probable because the defect in starch digestion disappeared when refected rats were fed an adequate diet containing yeast as the source of vitamin B; and because, conversely, the starch defect could not be induced in rats fed a similarly complete diet. Further, the body weight of normally growing refected rats decreased rapidly when they received a diet devoid of starch, suggesting an essential connection between the production of vitamin B in refected rats and the defective starch digestion. Young rats, likewise, on a starch-free diet did not become refected when fed with white feces, yet they declined and died as soon as the supplement was withdrawn. The rapid growth of control rats receiving a supplement of yeast in addition to the starch-free diet showed that this diet was adequate for growth in spite of its lack of starch. Apparently, the ability to grow and the defective digestion of starch were in some unknown way closely related.

In intimations upon the cause of the condition, Fridericia and associates stated that the feces and the content of the cecum of refected rats contained a virus which was responsible for refection. The virus could be kept in a dry state in feces for 5½ months without deterioration; it was not destroyed by heat at 80° for ten minutes; but it was impaired by heat at 100° for the same length of time. It is not amiss to recall Eijkman's theory that polyneuritis in rice-fed chickens was due to an intoxication arising from intestinal fermentation of starch in the food. Although this theory has long been abandoned, Fridericia and his coworkers point out that the phenomenon of refection in an unexpected way corroborates Eijkman's ideas; for in refected rats the digestion of starch is defective.

That refection did appear among rats on a diet lacking in vitamin B was confirmed by Roscoe (263a). She also observed that the phenomenon appeared in rats receiving one component of the vitamin but not the other. For example, rats fed Peters' yeast extract, which contains the antineuritic vitamin but not the thermostable factor P-P, ordinarily cease to grow, and after five to ten weeks develop skin-lesions. In one instance where the skin-lesions were well developed and had been present for two weeks, the skin healed spontaneously and normal growth ensued without change of ration.

On the other hand, inclusion in a basal diet of autoclaved yeast, which contains the P-P factor but not the antineuritic vitamin, usually leads to loss of appetite, decline in weight, often paralysis, and finally

death. After one to two weeks of deprivation of the antineuritic vitamin, the animals became refected, prevention of neuritic symptoms was complete, and normal growth was recorded. From these data Roscoe concluded that refected rats could dispense with either of the two components of vitamin B, since normal growth and health ensued after refection.

If cognizance of the multiple nature of vitamin B has contributed nothing else, it has afforded an insight into two perplexities which have harassed investigators in this field for some time. Of these Goldberger has spoken: "It is, at least, possible that in the past, workers in discarding 'inactive' fractions have unwittingly been throwing away the very thing they were laboriously seeking. This may perhaps explain, at least in part, the somewhat unaccountable losses of vitamin in the process of fractionation of 'active' preparations" (236). True, while a recognition of the multiplicity has cast this light, it has likewise thrown a pall over the vitamin B problem. Where there was one substance awaiting isolation, there are now—no one knows how many. At present there is no clarity in regard to the relation of the various essential components described by investigators; for instance, whether all the thermostable factors are identical, or whether the alkali-stable factor is the same as a thermostable factor. Moreover, Funk's contention that bios, as an essential factor for animal growth, is related to vitamin B must not be forgotten. Particularly does this contingency present difficulties when it is remembered that bios itself has been separated into three fractions. But if these developments, which have revealed the multiple nature of vitamin B, have increased the work necessary before chemical isolation and identification can be achieved, they have likewise increased the interest in this direction. And almost forty years after the discovery of vitamin B finds investigators immersed in the problem with added zeal.

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Addendum: Since the preparation of this review, quite a number of articles upon vitamin B have appeared in the literature. Most of these papers present further observations upon the existence of two vitamins, B and F; others advance the claim of a third independent factor. Of necessity, only the references can be given.

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